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<p>(54) Title: METHODS AND COMPOSITIONS FOR GENE, TUMOR, AND VIRAL INFECTION THERAPY, AND PREVENTION OF PROGRAMMED CELL DEATH (APOPTOSIS)</p> <p>(57) Abstract</p> <p>The present invention relates to methods of treatment of programmed cell death (apoptosis) through the use of the HSV-1 gene $\gamma_134.5$ or the product of its expression, ICP34.5. The gene and its expression have been demonstrated to be required for HSV-1 neurovirulence, and in particular, to act as an inhibitor of neuronal programmed cell death which allows for viral replication. Use of the gene therapy, or the protein itself, can be expected to result in inhibition of programmed cell death in various neurodegenerative diseases. This invention also relates to novel vectors for gene therapy, including modified herpes virus. Methods are presented for conducting assays for substances capable of mimicing, potentiating or inhibiting the expression of $\gamma_134.5$ or the activity of ICP34.5. Also, methods are disclosed for the treatment of tumorigenic diseases, including cancer, and for treatment of herpes and other viral infections using inhibitors of $\gamma_134.5$ expression or ICP34.5 activity.</p>			

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METHODS AND COMPOSITIONS FOR GENE, TUMOR, AND VIRAL INFECTION THERAPY, AND PREVENTION OF PROGRAMMED CELL DEATH (APOPTOSIS)

BACKGROUND OF THE INVENTION

The government may own certain rights in the present invention pursuant to grants from the National Cancer Institute (CA47451) and from the National Institute for 5 Allergy and Infectious Diseases (AI24009 and AI1588), and the United States Public Health Service.

1. Field of the Invention

The present invention is directed to methods for blocking or delaying programmed cell death, for delivery of 10 gene therapy to specific cells, and for treatment of cancer and other tumorigenic diseases, as well as treatment of viral infections, through the potentiation of programmed cell death in tumor or viral host cells. The present invention is also directed to assays for candidate 15 substances which can either inhibit, or potentiate programmed cell death.

2. Description of the Related Art

a. Programmed Cell Death (Apoptosis)

In the last decade there has been increasing 20 acceptance in the scientific community of the idea that cells may actually be internally programmed to die at a certain point in their life cycle. As an active cellular mechanism programmed cell death, or apoptosis, has several important implications. First, it is clear that such an 25 active process can provide additional means of regulating cell numbers as well as the biological activities of cells. Secondly, mutations or cellular events which potentiate apoptosis may result in premature cell death. Third, a form of cell death which is dependent on a specific active 30 cellular mechanism can at least potentially be suppressed.

Finally, an inhibition of preprogrammed cell death would be expected to lead to aberrant cell survival and could be expected to contribute to oncogenesis.

In general, apoptosis involves distinctive morphological changes including nuclear condensation and degradation of DNA to oligonucleosomal fragments. In certain circumstances it is evident that apoptosis is triggered by or is preceded by changes in protein synthesis. Apoptosis appears to provide a very clean process for cellular destruction, in that the cells are disposed of by specific recognition and phagocytosis prior to bursting. In this manner cells can be removed from a tissue without causing damage to the surrounding cells. Thus, it can be seen that programmed cell death is crucial in a number of physiological processes, including morphological development, clonal selection in the immune system, and normal cell maturation and death in other tissue and organ systems.

It has also been demonstrated that cells can undergo apoptosis in response to environmental information. Examples include the appearance of a stimulus, such as glucocorticoid hormones for immature thymocytes, or the disappearance of a stimulus, such as interleukin-2 withdrawal from mature lymphocytes, or the removal of colony stimulating factors from hemopoietic precursors (for a review of literature see Williams, Cell, 85; 1097-1098, June 28, 1991). Furthermore, it has recently been demonstrated that the response of removal to nerve growth factor from established neuronal cell cultures mimics target removal, or axotomy, or other methods of trophic factor removal, and it has been postulated that the cellular mechanism involved in this response is a triggering of a suicide program or programmed cell death following the nerve growth factor removal. (See Johnson et al., Neurobiol. of Aging, 10: 549-552, 1989). The authors

propose a "death cascade" or "death program", which envisions that trophic factor deprivation initiates the transcription of new mRNA and the subsequent translation of that mRNA into death associated proteins which act in sequence to ultimately produce "killer proteins". Such an intracellular mechanism seems to fit well with the characteristics of apoptosis discussed above, eg., death of specific cells without the release of harmful materials and without the disruption of tissue integrity. Furthermore, the authors indicate that inhibitors of macromolecular synthesis prevented the death of neurons in the absence of nerve growth factor.

Studies have been conducted to explore the possibility that tumor cells could be eliminated by artificially triggering apoptosis. The APO-1 monoclonal antibody can induce apoptosis in several transformed human B and T cell lines. The antibody binds to a surface protein and could act either by mimicking a positive death-inducing signal or by blocking the activity of a factor required for survival. Also, anti-FAS antibodies have similar effects, and the recent cloning and sequencing of the gene for the FAS antigen has shown that it is a 63 kilodalton transmembrane receptor. Itoh et al., Cell 66: 233-243 (1991).

However, it is important to note that neither APO-1 nor FAS can function exclusively as triggers for cell death. Both are cell surface receptors that may activate quite different responses under other circumstances. Moreover, these antigens are not confined to tumor cells and their effect on normal cells is certainly an important consideration, as is the possible appearance of variants that no longer display the antigens.

It has also been demonstrated that the cell death induced by a range of cytotoxic drugs, including several used in cancer therapy, has also been found to be a form of

apoptosis. In fact, the failure of apoptosis in tumor cells could be of fundamental importance in contributing not only to the evasion of physiological controls on cell numbers, but also to resistance both to natural defenses 5 and to clinical therapy.

It has also been demonstrated that expression of the bcl-2 gene can inhibit death by apoptosis. The bcl-2 gene was isolated from the breakpoint of the translocation between chromosomes 14 and 18 found in a high proportion of 10 the most common human lymphomas, that being follicular B cell lymphomas. The translocation brings together the bcl-2 gene and immunoglobulin heavy chain locus, resulting in an aberrantly increased bcl-2 expression in B cells. Subsequently, Henderson et al. (Cell, 65: 1107-1115, 1991) 15 demonstrated that expression of latent membrane protein 1 in cells infected by Epstein-Barr virus protected the infected B cells from programmed cell death by inducing expression of the bcl-2 gene. Sentman et al. (Cell, 67: 879-88, November 29, 1991) demonstrated that expression of 20 the bcl-2 gene can inhibit multiple forms of apoptosis but not negative selection in thymocytes, and Strasser et al. (Cell, 67: 889-899, November 29, 1991) demonstrated that expression of a bcl-2 transgene inhibits T cell death and can perturb thymic self- censorship. Clem et al. (Science, 25 245: 1388-1390, November 29, 1991) identified a specific baculovirus gene product as being responsible for blocking apoptosis in insect cells.

b. Herpes Virus Infections and Neurovirulence

The family of herpes virus includes animal viruses of 30 great clinical interest because they are the causative agents of many diseases. Epstein-Barr virus has been implicated in B cell lymphoma; cytomegalovirus presents the greatest infectious threat to AIDS patients; and Varicella Zoster Virus, is of great concern in certain parts of the

world where chicken pox and shingles are serious health problems. A worldwide increase in the incidence of sexually transmitted herpes simplex (HSV) infection has occurred in the past decade, accompanied by an increase in 5 neonatal herpes. Contact with active ulcerative lesions or asymptomatically excreting patients can result in transmission of the infectious agent. Transmission is by exposure to virus at mucosal surfaces and abraded skin, which permit the entry of virus and the initiation of viral 10 replication in cells of the epidermis and dermis. In addition to clinically apparent lesions, latent infections may persist, in particular in sensory nerve cells. Various stimuli may cause reactivation of the HSV infection. Consequently, this is a difficult infection to eradicate. 15 This scourge has largely gone unchecked due to the inadequacies of treatment modalities.

The known herpes viruses appear to share four significant biological properties:

1. All herpes viruses specify a large array of 20 enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase, etc.), DNA synthesis (e.g., DNA polymerase helicase, primase), and, possibly, processing of proteins (e.g., protein kinase), although the exact array 25 of enzymes may vary somewhat from one herpesvirus to another.

2. Both the synthesis of viral DNAs and the assembly of capsids occur in the nucleus. In the case of some 30 herpes viruses, it has been claimed that the virus may be de-enveloped and re-enveloped as it transits through the cytoplasm. Irrespective of the merits of these conclusions, envelopment of the capsids as it transits through the nuclear membrane is obligatory.

3. Production of infectious progeny virus is invariably accompanied by the irreversible destruction of the infected cell.

4. All herpes viruses examined to date are able to
5 remain latent in their natural hosts. In cells harboring latent virus, viral genomes take the form of closed circular molecules, and only a small subset of viral genes is expressed.

Herpes viruses also vary greatly in their biologic properties. Some have a wide host-cell range, multiply efficiently, and rapidly destroy the cells that they infect (e.g., HSV-1, HSV-2, etc.). Others (e.g., EBV, HHV6) have a narrow host-cell range. The multiplication of some herpes viruses (e.g., HCMV) appears to be slow. While all herpes viruses remain latent in a specific set of cells, the exact cell in which they remain latent varies from one virus to another. For example, whereas latent HSV is recovered from sensory neurons, latent EBV is recovered from B lymphocytes. Herpes viruses differ with respect to 20 the clinical manifestations of diseases they cause.

Herpes simplex viruses 1 and 2 (HSV-1, HSV-2), are among the most common infectious agents encountered by humans (Corey and Spear, N. Eng. J. Med., 314: 686-691, 1986). These viruses cause a broad spectrum of diseases 25 which range from mild and nuisance infections such as recurrent herpes simplex labialis, to severe and life-threatening diseases such as herpes simplex encephalitis (HSE) of older children and adults, or the disseminated infections of neonates. Clinical outcome of herpes 30 infections is dependent upon early diagnosis and prompt initiation of antiviral therapy. However, despite some successful therapy, dermal and epidermal lesions recur, and HSV infections of neonates and infections of the brain are associated with high morbidity and mortality. Earlier

diagnosis than is currently possible would improve therapeutic success. In addition, improved treatments are desperately needed.

Extrinsic assistance has been provided to infected
5 individuals, in particular, in the form of chemicals. For example, chemical inhibition of herpes viral replication has been effected by a variety of nucleoside analogues such as acyclovir, 5-fluorodeoxyuridine (FUDR), 5-iododeoxyuridine, thymine arabinoside, and the like.

10 Some protection has been provided in experimental animal models by polyspecific or monospecific anti-HSV antibodies, HSV-primed lymphocytes, and cloned T cells to specific viral antigens (Corey and Spear, N. Eng. J. Med., 314: 686-691, 1986). However, no satisfactory treatment
15 has been found.

The $\gamma_{134.5}$ gene of herpes simplex virus maps in the inverted repeat region of the genome flanking the L component of the virus. The discovery and characterization of the gene was reported in several articles (Chou and
20 Roizman, J. Virol., 57: 629-635, 1986, and J. Virol., 64: 1014-1020, 1990; Ackermann et al., J. Virol., 58: 843-850, 1986). The key features are: (i) the gene encodes a protein of 263 amino acid in length; (ii) the protein contains Ala-Thr-Pro trimer repeat ten times in the middle
25 of the coding sequence; (iii) the protein is basic in nature and consists of large number of Arg and Pro amino acids; (iv) the promoter of the gene maps in the a sequence of the genome which also serves several essential viral functions for the virus; (v) the *cis*-acting element
30 essential for the expression of the gene $\gamma_{134.5}$ is contained within the a sequence, in particular, the DR2 (12 base pair sequence repeated 22 times) and U_b element. This type of promoter structure is unique to this gene and not shared by other viral gene promoters.

The function of the gene $\gamma_134.5$ in its ability to enable the virus to replicate, multiply and spread in the central nervous system (CNS) was demonstrated by a set of recombinant viruses and by testing their abilities to cause 5 fatal encephalitis in the mouse brain. The mutant viruses lacking the gene therefore lost their ability to multiply and spread in the CNS and eyes and therefore is non-pathogenic. See Chou et al., Science, 250: 1212-1266, 1990.

10 The $\gamma_134.5$ gene functions by protecting the nerve cells from total protein synthesis shutoff in a manner characteristic of programmed cell death (apoptosis) in neuronal cells. The promoter appears to contain stress response elements and is transactivated by exposure to UV 15 irradiation, viral infection, and growth factor deprivation. These data suggest that the gene $\gamma_134.5$ is transactivated in the nerve cells at times of stress to prevent apoptosis.

20 The significance of these findings therefore lies in the fact that $\gamma_134.5$ extends viability or lends protection to the nerve cells so that in this instance, the virus can replicate and spread from cell to cell -- defined as neurovirulence. It also appears that the protection can be extended to other toxic agents or environmental stresses to 25 which the cell is subjected. An important aspect about the nature of the neurons, unlike any other cells in human, is the fact that neurons in the brain, eyes or CNS do not regenerate which forms the basis of many impaired neurological diseases. Any genes or drugs that extend the life of cells from death or degeneration can be expected to 30 have a significant impact in the area of neural degeneration.

The role of $\gamma_134.5$, and anti-apoptosis factors, in infected cells is in its early stages of elucidation.

Recent studies have suggested that Epstein-Barr virus enhances the survival capacity of infected cells through latent membrane protein 1(LMP1)-induced up-regulation of bcl-2. In that system it is postulated that LMP 1 induced
5 bcl-2 up regulation gives virus infected B cells the potential to by-pass physiological selection and gain direct access to long lived memory B cell pools. However, bcl-2 expression fails to suppress apoptosis in some situations, for example upon withdrawal of interleukin-2 or
10 interleukin-6. Moreover, the intracellular mechanism of action of bcl-2 expression remains unknown.

c. Programmed Cell Death and Disease Therapy

In light of the foregoing, it is apparent that the expression of γ ,34.5 in CNS cells added an extra dimension of protection to the neurons against viral infection, and naturally occurring and stress-induced apoptosis. An appreciation of this extra dimension of protection can be utilized in novel and innovative means for control and treatment of central nervous system (CNS) disorders.
15 Specifically, treatment of CNS degenerative diseases, including Alzheimer's disease, Parkinson's disease, Lou Gerig's disease, and others the etiology of which may be traceable to a form of apoptosis, and the treatment of which is currently very poor, could be improved
20 significantly through the use of either the γ ,34.5 gene in gene therapy or the protein expressed by γ ,34.5 as a therapeutic agent. This is especially critical where the death of neuronal cells is involved, due to the fact that, as noted, such cells do not reproduce post-mitotically.
25 Since a finite number of neurons are available it is crucial to have available methods and agents for their protection and maintenance. γ ,34.5 is also a very useful gene for assays of substances which mimic the effect of γ ,34.5 and block stress of biologically induced programmed
30 cell death.
35

Furthermore, the HSV-1 virus, appropriately modified so as to be made non-pathogenic, can serve as a vehicle for delivery of gene therapy to neurons. The HSV-1 virus is present in neurons of the sensory ganglia of 90% of the world's human population. The virus ascends into neuronal cell bodies via retrograde axonal transport, reaching the axon from the site of infection by the process of neurotropism. Once in the neuronal cell body the virus remains dormant until some form of stress induces viral replication (e.g. UV exposure, infection by a second virus, surgery or axotomy). As noted, the use of HSV-1 as a vector would necessitate construction of deletion mutants to serve as safe, non-pathogenic vectors. Such a virus would act as an excellent vector for neuronal gene therapy and its use would be an especially important development since few methods of gene therapy provide a means for delivery of a gene across the central nervous system's blood-brain barrier.

Moreover, other viruses, such as HSV-2, picornavirus, coronavirus, eunyavirus, togavirus, rhabdovirus, retrovirus or vaccinia virus, are available as vectors for γ ,34.5 gene therapy. As discussed with regard to the use of HSV-1 viruses, these vectors would also be altered in such a way as to render them non-pathogenic. In addition to the use of an appropriately mutated virus, implantation of transfected multipotent neural cell lines may also provide a means for delivery of the γ ,34.5 gene to the CNS which avoids the blood brain barrier.

In addition, use of the HSV-1 virus with a specific mutation in the γ ,34.5 gene provides a method of therapeutic treatment of tumorogenic diseases both in the CNS and in all other parts of the body. The " γ ,34.5 minus" virus can induce apoptosis and thereby cause the death of the host cell, but this virus cannot replicate and spread. Therefore, given the ability to target tumors within the CNS, the γ ,34.5 minus virus has proven a powerful

therapeutic agent for hitherto virtually untreatable forms of CNS cancer. Furthermore, use of substances, other than a virus, which inhibit or block expression of genes with anti-apoptotic effects in target tumor cells can also serve
5 as a significant development in tumor therapy and in the treatment of herpes virus infection, as well as treatment of infection by other viruses whose neurovirulence is dependent upon an interference with the host cells' programmed cell death mechanisms.

10

SUMMARY OF THE INVENTION

This invention relates to methods for the prevention or treatment of programmed cell death, or apoptosis, in neuronal cells for therapy in connection with neurodegenerative diseases, as well as methods of treatment
15 of cancer and other tumorogenic diseases and herpes virus infection. The present invention also relates to assay methodologies allowing for the identification of substances capable of modulating the effects of the γ ,34.5 gene or its protein expression product ICP34.5, i.e., substances
20 capable of potentiating or inhibiting their effects. Additionally, the present invention also relates to assay methodologies designed to identify candidate substances able to mimic either γ ,34.5 expression or the activity of ICP34.5. The present invention also relates to methods of
25 delivering genes to cells for gene therapy.

In one illustrative embodiment of the present invention a method of preventing or treating programmed cell death in neuronal cells is described in which a non-pathogenic vector is prepared which contains the γ ,34.5 gene. This vector is then introduced into neuronal cells
30 which are presently undergoing or are likely to undergo programmed cell death. Those skilled in the art will realize that several vectors are suitable for use in this method, although the present invention envisions the use of

certain unique and novel vectors designed specifically for use in connection with delivery of the $\gamma_{134.5}$ gene.

One such vector envisioned by the present invention is the HSV-1 virus itself, modified so as to render it non-pathogenic. Because of the unique capability of the HSV-1 virus to use an axon's internal transport system to move from the peripheral nerve endings of the neuron into the neuronal cell body, the present invention proposes the use of the non-pathogenic HSV-1 virus injected into the vicinity of the synaptic terminals of affected neurons, or in the area of a peripheral wound or lesion or other appropriate peripheral locus. The HSV-1 virus containing the $\gamma_{134.5}$ gene, under a different target-specific promoter, would then be transported into the neuronal cell body via retrograde axonal transport.

The present invention envisions specific genomic modifications being introduced into the HSV-1 virus in order to render the virus non-cytotoxic. These modifications could include deletions from the genome, rearrangements of specific genomic sequences, or other specific mutations. One example of such a modification comprises modification or deletion of the $\alpha 4$ gene which encodes the ICP4 protein. Deletion or modification of the gene expressing ICP4 renders the HSV-1 virus unable to express genes required for viral DNA and structural protein synthesis. However, the $\gamma_{134.5}$ gene placed under a suitable promoter would be expressed, thus inducing an anti-apoptotic effect in the neuron without the potential for stress induced neurovirulence. Other genes which might be modified include the the $\alpha 0$ gene. The present invention also envisions the use of other vectors including, for example, retrovirus, picorna virus, vaccinia virus, HSV-2, coronavirus, eunyavirus, togavirus or rhabdovirus vectors. Again, use of such viruses as vectors will necessitate

construction of deletion mutations so that the vectors will be safe and non-pathogenic.

Another method by which the present invention envisions introducing the γ ,34.5 gene into neuronal cells undergoing or likely to undergo programmed cell death, is through the use of multi-potent neural cell lines. Such lines have been shown to change phenotype *in vitro* and have also been demonstrated to become integrated into the central nervous system of mice and to differentiate into neurons or glia in a manner appropriate to their site of engraftment. Snyder, et al., Cell, 68: 33-51, 1992. Transplant or engraftment of multi-potent neural cell lines into which the γ ,34.5 gene has been introduced into an area of the central nervous system in which cells are undergoing or are likely to undergo programmed cells death is expected to lead to reversal and inhibition of programmed cell death.

It is expected that the ability of γ ,34.5 to inhibit apoptosis will be a boon not only in human medicine, but also in basic scientific research. In this regard the present invention also envisions the use of the γ ,34.5 gene in the extension of the life of neuronal cells in cell culture. Introduction of a non-cytotoxic vector into cultured neuronal cells will have an anti-apoptotic effect and will thereby extend the life of cell cultures. This in turn will extend the time periods over which experimentation may be conducted, and can also be expected to decrease the cost of conducting basic research.

In addition to utilizing a vector comprising the γ ,34.5 gene, the present invention also discloses a method of preventing or treating programmed cell death in neuronal cells which involves the use of the product of expression of the γ ,34.5 gene. The protein expressed by γ ,34.5 is called ICP34.5. Ackermann, et al. (J. Virol., 58: 843-850,

1986) reported that ICP34.5 has an apparent molecular weight of 43,500 upon SDS-polyacrylamide gel electrophoresis, appears to accumulate largely in the cytoplasm of HIV infected cells, and in contrast to many 5 HSV-1 proteins, ICP34.5 has been demonstrated to be soluble in physiologic solutions.

In practicing this method or the method in which the γ ,34.5 gene is introduced into cells, it is envisioned that the γ ,34.5 gene or a biological functional equivalent thereof could be used for gene therapy, or ICP34.5 in a purified form or a biological functional equivalent of the ICP34.5 protein could be utilized as an anti-apoptotic agent. As used herein, functional equivalents are intended to refer to those proteins, and their encoding 10 nucleic acid sequences, in which certain structural changes have been made but which nonetheless are, or encode, 15 proteins evidencing an effect similar to that of ICP34.5.

In light of the fact that certain amino acids may be substituted for other amino acids in a protein without appreciable loss of defined functional activity, it is contemplated by the inventors that various changes may be made in the sequence of the ICP34.5 protein (or the underlying DNA of the γ ,34.5 gene) without an appreciable loss of biological utility or activity. Amino acids with 20 similar hydropathic scores may be substituted for one another (see Kyte et al., J. Mol. Biol., 157: 105-132, 1982, incorporated herein by reference), as may amino acids with similar hydrophilicity values, as described in U.S. 25 Patent 4,554,101, incorporated herein by reference.

Therefore, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Examples 30 substitutions which take various of the foregoing

characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

5 This embodiment of the present invention describes a method which involves combining ICP34.5 or a biological functional equivalent thereof with a pharmaceutically acceptable carrier in order to form a pharmaceutical composition. (It should be understood in subsequent
10 discussions that when γ 34.5 or ICP34.5 are referred to, the inventors intend to include biological functional equivalents, including any chemicals which mimic the effect of γ 34.5.) Such a composition would then be administered to neurons likely to undergo or undergoing programmed cell
15 death. Such a composition could be administered to an animal using intravenous, intraspinal injection or, in certain circumstances, oral, intracerebral or intraventricular administration may be appropriate. Furthermore, neuronal cells in culture could also benefit
20 from administration of ICP34.5 through administration directly into the medium in which the neuronal cells are grown.

ICP34.5 can be prepared using a nucleic acid segment which is capable of encoding ICP34.5 (i.e., the γ 34.5 gene or a biological functional equivalent). Such a segment could be expressed using, for example, a technique involving transferring the γ 34.5 segment into a host cell, culturing the host cell under conditions suitable for expression of the segment, allowing expression to occur,
25 and thereafter isolating and purifying the protein using well established protein purification techniques. The nucleic acid segment would be transferred into host cells by transfection or by transformation of a recombinant vector into the host cell.

A particularly important embodiment of the present invention relates to assays for candidate substances which can either mimic the effects of the γ 134.5 gene, or mimic the effects of ICP34.5, as well as assays for candidate substances able to potentiate the function of γ 134.5 or potentiate the protective function of ICP34.5. Additionally, methods for assaying for candidate substances able to inhibit either γ 134.5 expression or the activity of ICP34.5 are also embodiments of the present invention.

In an exemplary embodiment, an assay testing for candidate substances which would block the expression of the anti-apoptosis gene or inhibit the activity of an anti-apoptotic protein such as ICP34.5 would proceed along the following lines. A test plasmid construct bearing the α sequence promoter and portions of the coding sequence of γ 134.5 is fused to the lacZ reporter gene, or any other readily assayable reporter gene. This construct is then introduced into an appropriate cell line, for example a neuroblastoma or PC12 cell line, by G418 selection. A clonal and continuous cell line for screening purposes is then established. A control plasma construct bearing an HSV late promoter (a promoter which would normally not be expressed in cell lines and not induced to express by a stress factor which would normally induce apoptosis) is fused to the same or different indicator gene. This construct is also introduced into a continuous clonal cell line and serves as a control for the test cell line. The anti-apoptosis drugs would then be applied. Environmental stresses which typically trigger α sequence promoter activation and cause programmed cell death, such as UV injury, viral infection or deprivation of nerve growth factor, would then be applied to the cells. In control cells, the stress should have no effect on the cells and produce no detectible reaction in the assay. Stress in a test cell line in the absence of a positive candidate substance would give rise to an appropriate reaction,

typically a colorimetric reaction. Introduction of stress to the test cell line in the presence of the candidate substance would give rise to an opposite colorimetric reaction indicating that the candidate substance 5 interferes either with expression of the $\gamma_{134.5}$ gene, or with the ability of the substance to interfere with the anti-apoptotic activity of ICP34.5.

Similarly, the present invention describes an assay for candidate substances which would mimic or potentiate 10 the activity of ICP34.5, or which would mimic the expression of $\gamma_{134.5}$, and such an assay would proceed along lines similar to those described above. A test cell line (e.g., a neuroblastoma cell line) constitutively expressing ICP34.5 and a fluorescent tagged cellular gene or any other 15 tag providing an easily detected marker signalling viability of the cells is produced. In addition, a corresponding null cell line consisting of an appropriate indicator gene, for example the α -lacZ indicator gene, and the same host indicator gene as in the test cell line is 20 also produced. Also, a third cell line (e.g., a vero cell line) consisting of the same indicator gene and the identical host indicator gene is also produced. Again, environmental stresses which trigger programmed cell death in the absence of $\gamma_{134.5}$ are applied to the cells. 25 Candidate substances are also applied in order to determine whether they are able to mimic or potentiate the anti-apoptotic effects of $\gamma_{134.5}$ expression or the anti-apoptotic activity of ICP34.5 or biological functional equivalents thereof.

30 The present invention also embodies a method of delivering genes for gene therapy. In an exemplary embodiment, the method involves combining the gene used for gene therapy with a mutated virus such as those described above, or with the HSV-1 virus rendered non-pathogenic. 35 The gene and the virus are then combined with a

pharmacologically acceptable carrier in order to form a pharmaceutical composition. This pharmaceutical composition is then administered in such a way that the mutated virus containing the gene for therapy, or the HSV-1 5 wild type virus containing the gene, can be incorporated into cells at an appropriate area. For example, when using the HSV-1 virus, the composition could be administered in an area where synaptic terminals are located so that the virus can be taken up into the terminals and transported in 10 a retrograde manner up the axon into the axonal cell bodies via retrograde axonal transport. Clearly, such a method would only be appropriate when cells in the peripheral or central nervous system were the target of the gene therapy.

The present invention also envisions methods and 15 compositions for the treatment of cancer and other tumorogenic diseases, as well as herpes infections or other infections involving viruses whose virulence is dependent upon an anti-apoptotic effect. Candidate substances identified as having an inhibiting effect upon either the 20 expression or activity of ICP34.5 identified in the assay methods discussed above could be used to induce cell death in target tumor cells, or in virus-infected cells. Pharmaceutical compositions containing such substances can be introduced using intrathecal, intravenous, or direct 25 injection into the tumor or the infected area, as appropriate.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows DNA sequence comparisons of HSV-1 strains F (SEQ ID NO's 1-5), 17syn+ (SEQ ID NO's: 6-9), MGH-10 (SEQ 30 ID NO's 10-15), and CVG-2 (SEQ ID NO's 16-20) in the region of the gene for ICP34.5 (left panel) and the predicted open frames for ICP34.5 in these strains (SEQ ID NO's 25-34) (right panel). Unless otherwise indicated by a new base (insertion of A, C, G, or T), a new amino acid (three-letter code), or absence of a base or amino acid (-), the 35

sequences for strains HSV-1(17)synt, HSV-1(MGH-10), and HSV-1(CVG-2) were identical to the sequence for HSV-1(F). An asterisk indicates initiation of a repeat sequence of nine nucleotides or three amino acids. Direct repeat 1(DR1) designates the 20-base-pair repeat sequence flanking the a sequence. Sequences upstream of direct repeat 1 are contained within the a sequence. The number at the end of each line indicates the relative position from nucleotide 1 (left panel) or amino acid 1 (right panel). The initiation and termination codons for the HSV-1(F) sequence are underlined.

Fig. 2 shows sequence arrangements of the genome of wild-type strain HSV-1 strain F [HSV-1(F)] and of recombinant viruses derived from it. Top line, the sequence arrangement of HSV-1(F) Δ305. The rectangles identify the inverted repeats ab, b' a' c, and ca. The HSV-(F) a sequence is present in a direct orientation at the two genomic termini and in the inverted orientation at the junction between the long and short components. The b and c sequences are approximately 9 and 6 kbp long, respectively. The triangle marked TK identifies the position of the tk gene and of the Bgl II to Sac I sequence of BamHI Q fragment deleted from HSV-1(F)Δ305. Lines two and three from the top show that the b sequences contain the genes specifying ICP34.5 and ICP0 and, since b sequence is repeated in an inverted orientation, there are two copies of these genes per genome. The construction of the a24-tk fragment containing portions of the glycoprotein H gene has been described. Chou and Roizman, J. Virol., 57: 15 (1986); Ackerman et al., J. Virol., 58: 843 (1986); Chou and Roizman, J. Virol., 64: 1014 (1990). Line 7 shows a schematic diagram of the insertion of the oligonucleotide containing stop codons in all three reading frames. The plasmids pRB3615 and pRB2976 used in the construction of R4002 and R4004, respectively, were described elsewhere. Chou and Roizman, J. Virol., 57: 629 (1986) and J. Virol.,

64: 1014 (1990). To generate pRB3616, plasmid pRB143 was digested with BstEII and Stu I, blunt-ended with T4 polymerase, and religated. The asterisks designate nucleotides from vector plasmid that form cohesive ends with the synthesized oligomers (SEQ ID NO's 21-22). The insertion of the $\alpha 4$ epitope into the first amino acid of ICP34.5 (line 9) has been described, Chou and Roizman, J. Virol., 64: 1014 (1990), except that in this instance the sequence was inserted into both copies of the $\gamma_{134.5}$ gene (SEQ ID NO's 23, 24 and 34). The tk gene was restored in all recombinant viruses tested in mice. HSV-1(F)R (line 6) was derived from R3617 by restoration of the sequences deleted in $\gamma_{134.5}$ and tk genes. N, Be, S, and St are abbreviations for Nco I, BstEII, Sac I, and Stu I restriction endonucleases (New England Biolabs), respectively. The numbers in parentheses are the tk^+ version of each construct tested in mice.

Fig. 3 shows an autoradiographic image of electrophoretically separated digest of plasmid, wild-type, and mutant virus DNAs, transferred to a solid substrate and hybridized with labelled probes for the presence of $\gamma_{134.5}$ and tk genes. The plasmids or viral DNAs shown were digested with BamHI or, in the case of R4009 shown in lanes 10, with both BamHI and Spe I. The hybridization probes were the fragment Nco I to Sph I contained entirely within the coding sequences of $\gamma_{134.5}$ (left panel) and the BamHI Q fragment of HSV-1(F) (right panel). The probes were labeled by nick translation of the entire plasmid DNAs with [$\alpha-^{32}P$] deoxycytidine triphosphate and reagents provided in a kit (Du Pont Biotechnology Systems). The DNAs that were limit digested with BamHI (all lanes) or both BamHI and Spe I (left panel, lane 10) were electrophoretically separated on 0.8% agarose gels in 90 mM trisphosphate buffer at 40 V overnight. The DNA was then transferred by gravity to two nitrocellulose sheets sandwiching the gel and hybridized overnight with the respective probes. $\gamma_{134.5}$ maps in BamHI

S and SP fragments, which form a characteristic ladder of bands at 500-bp increments. The ladders are a consequence of a variable number of α sequences in the repeats flanking the unique sequences of the junction between the long and short components, whereas BamHI S is the terminal fragment of the viral genome at the terminus of the long component, whereas BamHI SP is a fragment formed by the fusion of the terminal BamHI S fragment with BamHI P, the terminal BamHI fragment of the short component. Bands of BamHI S, SP, and Q and their deleted versions, Δ BamHI S, Δ BamHI SP, and Δ BamHI Q (Δ Q), respectively, are indicated. Band 1 represents the 1.7-kbp α 27-tk insert into the BamHI SP fragment in R4002, and therefor this fragment reacted with both labeled probes (lanes 4). Band 2 represents the same insertion into the BamHI S fragment.

Fig. 4 shows autoradiographic images (left panel) and photograph of lysates of cells mock infected (M) or infected with HSV-1(F) and recombinant viruses (right panel) separated electrophoretically in denaturing polyacrylamide (10%) gels, transferred electrically to a nitrocellulose sheet, and stained with rabbit polyclonal antibody R4 described elsewhere. Ackerman et al., J. Virol., 58: 843 (1986); Chou and Roizman, J. Virol., 64: 1014 (1990). Replicate cultures of Vero cells were infected and labeled with [35 S]methionine (Du Pont Biotechnology Systems) from 12 to 24 hours after infection, and equivalent amounts of cell lysates were loaded in each slot. The procedures were as described (Ackerman et al.; Chou and Roizman) except that the bound antibody was made apparent with the alkaline phosphatase substrate system supplied by Promega, Inc. Infected cell proteins were designated by number according to Honess and Roizman (J. Virol., 12: 1346 (1973)). The chimeric ICP34.5 specified by R4003 migrated more slowly than the protein produced by other viruses because of the increased molecular weight caused by the insertion of the epitope.

Fig. 5 is a schematic representation of the genome structure and sequence arrangements of the HSV-1 strain F [HSV01(F)] and related mutants. Top line: The two covalently linked components of HSV-1 DNA, L and S, each consist of unique sequences flanked by inverted repeats (7, 31). The reiterated sequences flanking the L component designated as ab ad b'a' are each 9 kb in size, whereas the repeats flanking the S component are 6.3 kb in size (31). Line 2: expansion of portions of the inverted repeat sequences ab and b'a' containing the γ_1 34.5 and α 0 genes. Line 3: sequence arrangement and restriction endonuclease sites in the expanded portions shown in line 2. Open box represents the 20 bp direct repeat sequence (DR1), flanking the a sequence (26,27). Restriction site designations are N,- NcoI; Be,- BstEII; S,- SacI; St,- StuI. Line 4: the thin line and filled rectangle represent the transcribed and coding domains of the γ_1 34.5 gene (406). Vertical line, location of the transcription initiation sites of γ_1 34.5 and of α 0 genes. In the R3616 viral recombinant, one Kb was deleted between BstEII at 28th amino acid of γ_1 34.5 to StuI at the 3' terminus of the genes as shown. In HSV-1(F)R DNA, the sequences deleted from the γ_1 34.5 gene in R3616 were restored and therefore the virus could be expected to exhibit a wild-type phenotype. The R4009 recombinant virus DNA contains an in frame translation termination codons at the BstEII site. Vertical arrow on top points to the site of the stop codon insertion.

Fig. 6 shows an autoradiographic image of eletrophoretically separated lysates of infected cells labeled for 90 minutes with 35 S-methionine at stated time points. The SK-N-SH neuroblastoma and Vero cell lines were mock infected (M) or exposed at 37°C to 5 pfu of wild-type or mutant viruses in 6 well (Costar, Cambridge, Mass.) dishes. At 2 hours post exposure, the cells were overlaid with mixture 199 supplemented with 1% calf serum. At 5.5 and 11.5 hours post exposure of cells to viruses, replicate

infected 6 well cultures were overlaid with 1 ml of the 199v medium lacking unlabeled methionine but supplemented with 50 μ Ci of 35 S-methionine (specific activity >1,000 Ci/mmol, Amersham Co. Downers Grove, IL). After 90 minutes in labeling medium, the cells were harvested, solubilized in a buffer containing sodium dodecyl sulphate, subjected to electrophoresis on a denaturing 12% polyacrylamide gels crosslinked with N, N' Diallyltartardiamide, electrically transferred to nitrocellulose sheet and subjected to autoradiography as previously described (13). Infected cell polypeptides (ICP) were designated according to Honess and Roizman, J. Virol., 12: 1347-1365 (1973).

Fig. 7 shows autoradiographic images of labeled polypeptides electrophoretically separated in denaturing gels and photographs of protein bands made apparent by their reactivity with antibodies. The SK-N-SH neuroblastoma and Vero cells were either mock infected (M) or infected with 5 pfu of either R3616 or the parent HSV-1(F) per cell as described in the legend to Figure 6. The cultures were labeled for 1.5 hr before harvesting at 13th hr post exposure of cells to virus. Preparation of cell extracts, electrophoresis of the polypeptides, electric transfer of the separated polypeptides to a nitrocellulose sheet, and autoradiography were carried out as described elsewhere (Ackerman et al., J. Virol., 52: 108-118, 1984). The nitrocellulose sheets were reacted with the respective antibodies with the aid of kits from Promega, Inc. (Madison, WI) according to manufacturer's instruction. Monoclonal antibodies H1142 against α 27 and H725 against the product of the U_L26.5 gene were the generous gift of Lenore Pereira, University of California at San Francisco. The M28 monoclonal antibody to U_S11 protein and the rabbit polyclonal antibody R161 against viral thymidine kinase (β tk) were made to a specific peptide (M. Sarmiento and B.

Roizman, unpublished studies) in this laboratory. ICP designations were the same as noted before.

Fig. 8 shows an autoradiographic image of viral proteins expressed during infection on SK-N-SH neuroblastoma cell lines in the presence or absence of phosphonoacetate (PAA). Duplicate SK-N-SH neuroblastoma cell cultures were either treated with phosphonoacetate (300 µg/ml; Sigma Chemical Co., St. Louis, MO) starting at 1.5 hr prior to infection continuously until the termination of infection or left untreated. The cultures were either mock infected or exposed to 5 pfu of either HSV-1(F), R3616, R4009 and HSV-1(F)R at 5 pfu/cell. At 11.5 hours post exposure to virus, the cells were overlaid with medium containing 50 µCi of ³⁵S-methionine as described in legend to Figure 6. Polypeptide extraction, electrophoresis on 12% polyacrylamide gels crosslinked with N,N' Diallyltartardiamide, electrical transfer to nitrocellulose sheets and autoradiography were as described in the legend to Figure 6.

Fig. 9 shows viral DNA and RNA accumulation in infected SK-N-SH neuroblastoma and Vero cell cultures. Left panel: Photograph of ethidium bromide stained agarose gel containing electrophoretically separated BamHI digests of total DNAs extracted from mock-infected cells or cells infected with HSV-1(F), R3616, R4009 or HSV-1(F)R viruses. Right panel: Hybridization of electrophoretically separated RNA transferred to a nitrocellulose sheets probed with RNA sequences antisense to α47, U_s10 and U_s11 open reading frames. SK-N-SH neuroblastoma and Vero cells were either mock-infected or exposed to 5 pfu of HSV-1(F) or of R3616 per cell. Total DNAs were extracted from cells at 17 hr post infection by the procedure published by Katz et al., (J. Virol., 64: 4288-4295), digested with BamHI, electrophoresed in 0.8% agarose gel at 40V overnight and stained with ethidium bromide for visualization. For RNA

analysis, SK-N-SH neuroblastoma and Vero cells were either mock-infected or infected with R3616 and HSV-1(F) as described above. At 13 hrs post exposure of cells to virus the RNA was extracted by the procedure of Peppel and Baglioni (BioTechniques, 9: 711-712, 1990). The RNAs were then separated by electrophoresis on 1.2% agarose gel, transferred by gravity to a nitrocellulose sheet and probed with anti-sense RNA made from in vitro transcription of PRB3910 off T7 promoter using kit from Promega, Inc. according to manufacturer's instruction. α 47, U_S10 and U_S11 transcripts overlap in sequence and share the same 3' co-terminal sequence. McGeoch et al., J. Gen. Virol., 64: 1531-1574 (1988). The U_S10 transcript is of low abundance and not detected in this assay.

15 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
Introduction

The present invention relates to the use of the HSV-1 γ ₁34.5 gene, the ICP34.5 protein expressed by that gene, and derivatives of the protein which function in a similar manner as therapeutics for (neuronal) programmed cell death. The present invention also relates to the use of altered, non-pathogenic HSV-1 virus (as well as other viruses) as a vector for gene therapy. Other aspects of the present invention relate to assays for detecting candidate substances capable of acting as anti-apoptotic agents, as well as assays for detecting candidate substances able to induce programmed cell death in tumor cells. Additionally, the present invention also relates to methods for treating cancer and other tumorigenic diseases. Finally, the present invention also relates to the use of candidate substances capable of inactivating the γ ₁34.5 gene or ICP34.5 and thereby suppressing HSV-1 and other viral infections.

35 The wild-type HSV-1 genome (150 kilobase pairs) has two components, L and S, each possessing unique sequences

flanked by inverted repeats. The repeat sequences of the L component, designated *ab* and *b'a'*, are each 9 kilobase pairs, whereas the repeat sequences of the S component, designated *a'c'* and *ca*, are each 6.5 kilobase pairs.

5 Wadsworth et al., J. Virol., 15: 1487-1497 (1975). The shared *a* sequence, 500-base pairs long in HSV-1 strain F [HSV-1(F)], is present in one copy at the S component terminus and in one to several copies, in the same orientation, at the junction between L and S components.
10 The L and S components invert relative to each other such that the DNA extracted from virions or infected cells consists of four isomers differing solely in the orientation of the L and S components relative to each other. Hayward et al., Proc. Natl. Acad. Sci. USA, 72:
15 4243-4247 (1975). The *a* sequence appears to be a *cis*-acting site for inversions inasmuch as insertion of the *a* sequence elsewhere in the genome or deletion of the entire internal inverted repeat sequences (*b'a'c'*) leads to additional inversions or the loss of the ability of the L
20 and S components to invert, respectively. The *a* sequence was also shown to contain the *cis*-acting sites for the circularization of the genome after infection, for cleavage of the HSV genome from concatemers, and for encapsidation of the DNA.

25 HSV-1 genomes contain at least 73 genes whose expression is coordinately regulated and sequentially ordered in cascade fashion. The α genes are expressed first, followed by β , γ_1 and γ_2 genes. The differentiation among β , γ_1 and γ_2 genes is operationally based on the 30 effect of inhibitors of viral DNA synthesis. Whereas the expression of β genes is stimulated and that of γ_1 genes is only slightly reduced by inhibitors of viral DNA synthesis, the expression of γ_2 genes stringently requires viral DNA synthesis.

In the course of studies on the function of the a sequence, Chou and Roizman (Cell, 41: 803-811, 1985) noted that the chimeric structure consisting of the a sequence fused to the 5' transcribed, noncoding sequences of the thymidine kinase (TK) gene of HSV-1 was inducible in transferred cells and regulated as a γ_1 gene when inserted into the viral genome. This observation suggested that the terminus of the a sequence nearest the b sequence of the inverted repeats contained a promoter and the transcription initiation site of a gene whose structural sequences were located in the b sequences flanking the L component. Studies involving hybridization of labeled DNA probes to electrophoretically separated RNAs extracted from infected cells, and S1 nuclease analyses confirmed the existence of RNA transcripts initiating in the a sequence. Nucleotide sequence analyses revealed the presence of an open reading frame capable of encoding a protein 263 amino acids long. Chou and Roizman, J. Virol., 64: 1014-1020 (1990).

Previous studies have shown that each inverted repeat of the S component contains in its entirety a gene designated $\alpha 4$, whereas each of those of the L component contains in its entirety a gene designated $\alpha 0$. See, e.g., Mackem and Roizman, J. Virol., 44: 934-947 (1982). The putative gene identified on the basis of nucleotide sequence and analyses of RNA is also present in two copies per genome. Because of the overlap of the domain of this gene with the a sequence containing the *cis*-acting sites for inversion, cleavage of DNA from concatemers, and packaging of the DNA, it was of interest to identify and characterize the gene product. For this purpose, the observation that the nucleotide sequence predicted the presence in the protein of the amino acid triplet Ala-Thr-Pro repeated 10 times was utilized, and antibody to a synthetic peptide synthesized on the basis of this sequence reacted with a 43,500-apparent-molecular-weight HSV-1 protein. Ackerman et al., J. Virol., 58: 843-850, 1986.

The extent of variability of the open reading frame that encodes ICP34.5 was established by comparing the nucleotide sequences of three HSV-1 strains passaged a limited number of times outside a human host. Chou and Roizman (J. Virol., 64: 1014-1020, 1990) reported that the gene that specifies ICP34.5 contains 263 codons conserved in all three limited passage strains but not in the reported sequence of the HSV-1(17)syn+ strain. (FIG. 1) To ensure that the antibody to a predicted repeat sequence, Ala-Thr-Pro, reacted with ICP34.5 rather than with a heterologous protein with a similar repeat sequence, a short sequence of 45 nucleotides that encodes an epitope characteristic of another HSV-1 gene was inserted near the 5' terminus of the ICP34.5-coding domain. The recombinant virus expressed a protein with an appropriately slower electrophoretic mobility and which reacted with both the monoclonal antibody to the inserted epitope and rabbit antiserum to the Ala-Thr-Pro repeat element.

Studies of the identification of the genes associated with neurovirulence have repeatedly implicated DNA sequences located at or near a terminus of the long component of HSV-1 DNA. Thus, Centifanto-Fitzgerald et al. (J. Exp. Med., 155: 475, 1982) transferred, by means of a DNA fragment, a virulence marker from a virulent to an antivirulent strain of HSV-1. Deletion of genes located at one terminus of the long component of HSV-1 DNA contributed to the lack of virulence exhibited by a prototype HSV vaccine strain. Meignier et al., J. Infect. Dis., 158: 602 (1988). In other studies, Javier et al. (J. Virol., 65: 1978, 1987) and Thompson et al. (Virology, 172: 435, 1989) demonstrated that an HSV-1 x HSV-2 recombinant virus consisting largely of HSV-1 DNA but with HSV-2 sequences located at one terminus of the long component was avirulent; virulence could be restored by rescue with the homologous HSV-1 fragment. Taha et al. (J. Gen. Virol., 70: 705, 1989) described a spontaneous deletion mutant

lacking 1.5 kbp at both ends of the long component of a HSV-2 strain'. Because of heterogeneity in the parent virus population, the loss of virulence could not be unambiguously related to the specific deletion, although 5 the recombinant obtained by marker rescue was more virulent than the deletion mutant. In neither study was a specific gene or gene product identified at the mutated locus, and no gene was specifically linked to virulence phenotype.

Role of the γ ,34.5 Gene

10 To test the possible role of the product of the γ ,34.5 gene, ICP34.5, a series of four viruses (Fig. 2) were genetically engineered by the procedures of Post and Roizman (Cell, 25: 227, 1981, incorporated herein by reference).

15 1) Recombinant virus R4002 (Fig. 4, lane 3) contained the insertion of a thymidine kinase (tk) gene driven by the promoter of the α 27 gene (α 27-tk) in both copies of the ICP34.5 coding sequences. It was constructed by cotransfected rabbit skin cells with intact DNA of HSV-
20 1(F) Δ 305, a virus from which a portion of the tk gene was specifically deleted, with the DNA of plasmid pRB3615, which contains the α 27-tk gene inserted into the γ ,34.5 gene contained in the BamHI S fragment. Recombinants that were tk⁺ were then selected on human 143 thymidine kinase minus
25 (TK⁻) cells. The fragment containing the α 27-tk gene contains downstream from the tk gene: the 5' untranscribed promoter, the transcribed noncoding sequence, and the initiating methionine codon of the glycoprotein H gene. The BstEII site into which the α 27-tk fragment was inserted
30 is immediately upstream of the codon 29 of the γ ,34.5 open reading frame. As a consequence, the initiating codon of glycoprotein H was fused in frame and became the initiating codon of the truncated open reading frame of the γ ,34.5 gene

(Fig. 2, line 3). The recombinant selected for further study, R4002, was shown to contain the α 27-tk gene insert in both copies of γ 134.5 gene (Fig. 3, lanes 4) and specified only the predicted truncated product of the 5 chimeric γ 134.5 gene (Fig. 4, right panel, lane 3). The amounts of the native ICP34.5 protein detected in these and previous studies have been generally low. The chimeric genes formed by the fusion of the 5' transcribed noncoding region and the initiating codon of glycoprotein H in frame 10 with the truncated γ 134.5 gene were expressed far more efficiently than the native genes.

2) The recombinant virus R3617 (Fig. 2, line 5 from the top) lacking 1 kb of DNA in each copy of the γ 134.5 gene was generated by cotransfected rabbit skin cells with intact R4002 DNA and the DNA of plasmid pRB3616. In this plasmid, the sequences containing most of the coding domain of γ 134.5 has been deleted (Fig. 2, line 5 from top). The tk⁻ progeny of the transfection was plated on 143TK⁻ cells overlaid with medium containing bromodeoxy uridine (BrdU). This procedure selects tk⁻ viruses, and since the tk gene is present in both copies of the γ 134.5 gene, the selected progeny of the transfection could be expected to contain deletions in both copies. The selected tk⁻ virus designated as R3617 was analyzed for the presence of the deletion in both copies of the γ 134.5 gene. For assays of neurovirulence, the deletion in the native tk⁻ gene of R3617, which traces its origin from HSV-1(F) Δ 305, had to be repaired. This was done by cotransfection of rabbit skin cells with intact R3617 DNA and BamHI Q fragment containing the tk gene. The virus selected for tk⁺ phenotype in 143TK⁻ cells was designated R3616. This virus contains a wild-type BamHI Q fragment (Fig. 3, right panel, lane 6) and does not make ICP34.5 (Fig. 4, right panel).

3) To ascertain that the phenotype of R3616 indeed reflects the deletion in the γ 134.5 gene, the deleted

sequences were restored by cotransfected rabbit skin cells with intact R3617 DNA, the HSV-1(F) BamHI Q DNA fragment containing the intact tk gene, and the BamHI SP DNA fragment containing the intact γ 34.5 gene in the molar ratios of 1:1:10, respectively. Viruses that were tk⁺ were then selected in 143TK⁻ cells overlaid with medium containing hypoxanthine, aminopterin, and thymidine. The tk⁺ candidates were then screened for the presence of wild-type tk and γ 34.5 genes. As expected, the selected virus designated HSV-1(F)R (Fig. 2, line 6) contained a wild-type terminal long component fragment (compare Fig. 3, left panel, lanes 2, 7, and 8), and expressed ICP34.5 (Fig. 4, right panel, lane 6).

4) To eliminate the possibility that the phenotype of R3616 reflects deletion in cryptic open reading frames, a virus was constructed (R4010, Fig. 2, line 7 from the top) that contains translational stop codons in all three reading frames in the beginning of the ICP34.5 coding sequence. The 20-base oligonucleotide containing the translational stop codons and its complement sequence (Fig. 2) were made in an Applied Biosystems 380D DNA synthesizer, mixed at equal molar ratio, heated to 80°C, and allowed to cool slowly to room temperature. The annealed DNA was inserted into the HSV-1(F) BamHI S fragment at the BstEII site. The resulting plasmid pRB4009 contained a stop codon inserted in the beginning of the ICP34.5 coding sequence. The 20 nucleotide oligomer DNA insertion also contained a Spe I restriction site, which allowed rapid verification of the presence of the insert. To generate the recombinant virus R4010, rabbit skin cells were cotransfected with the intact DNA of R4002 and the pRB4009 plasmid DNA. Recombinants that were tk⁻ were selected in 143TK⁻ cells in medium containing BrdU. The tk⁺ version of this virus, designated R4009, was generated by cotransfection of intact tk⁻ R4010 DNA with HSV-1(F) BamHI Q DNA fragment, and selection of tk⁺ progeny. The virus selected for

neurovirulence studies, R4009, contained the Spe I restriction endonuclease cleavage site in both BamHI S and SP fragments (compare Fig. 3, left panel, lanes 9 and 10) and did not express ICP34.5 (Fig. 4, right panel, lane 7).

5 5) R4004 (Fig. 2, last line) was a recombinant virus produced by insertion of a sequence encoding 16 amino acids. This sequence has been shown to be the epitope of the monoclonal antibody H943 reactive with a viral protein designated as ICP4. Hubenthal-Voss et al., J. Virol., 62: 10 454 (1988). The virus was generated by cotransfected intact R4002 DNA and the DNA of plasmid pRB3976 containing the insert, and the selected tk⁻ progeny was analyzed for the presence of the insert. For neurovirulence studies, its tk gene was restored (recombinant virus R4003) as 15 described above. The DNA sequence was inserted in frame at the Nco I site at the initiating methionine codon of the γ₁34.5 gene. The insert regenerated the initiating methionine codon and generated a methionine codon between the epitope and the remainder of ICP34.5. Because of the 20 additional amino acids, the protein migrated more slowly in denaturing polyacrylamide gels (Fig. 4, right panel, lane 4).

Plaque morphology and size of all of the recombinants were similar to those of the wild-type parent, HSV-1(F) 25 when plated on Vero, 143TK⁻, and rabbit skin cells lines. Whereas HSV-1(F)R and R4003 replicated as well as the wild-type virus in replicate cultures of Vero cells, the yields of R3616 and R4009 were reduced to one-third to one-fourth the amount of the wild type. Although ICP34.5 was not 30 essential for growth of HSV-1 in cells in culture, the results of the studies shown in Table 1 indicate that the deletion or termination of translation of the γ₁34.5 has a profound effect on the virulence of the virus. Thus, all 35 of the mice inoculated with the highest concentration [1.2 x 10⁶

plaque-forming units (PFU)] of R3616 survived. In the case of R4009, only three of ten mice died as a result of inoculation with the highest concentration of virus ($\sim 10^7$ PFU). In comparison with other deletion mutants, R3616 and 5 R4009 rank among the least pathogenic viruses reported to date. The virus in which the $\gamma_134.5$ gene was restored exhibited the virulence of the parent virus.

TABLE 1

	Virus in the inoculum	Genotype	PFU/LD ₅₀
	HSV-1(F)	Wild-type parent virus	420
	R3616	1000-bp deletion in the $\gamma_134.5$	>1,200,000
15	HSV-1(F)R	Restoration of $\gamma_134.5$ and tk	130
	R4009	Stop codon in $\gamma_134.5$	>10,000,000
	R4003	Monoclonal antibody epitope inserted at the NH ₂ terminal	4,200

20 Comparative ability of wild-type and recombinant viruses to cause death after intracerebral inoculation of mice. The neurovirulence studies were done on female BALB/C mice obtained at 21 days of age (weight \pm 1.8 g) from Charles River Breeding Laboratories in Raleigh, North Carolina. The viruses were diluted in minimum essential medium containing Earle's salts and 10% fetal bovine serum, penicillin, and gentamicin. The mice were inoculated intracerebrally in the right cerebral hemisphere with a 26-gauge needle. The volume delivered was 0.03 ml, and each dilution of virus was tested in groups of ten mice. The animals were checked daily for mortality for 21 days. The LD₅₀ was calculated with the aid of the 25 "Dose effect Analysis" computer program from Elsevier Biosoft, Cambridge, United Kingdom.

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The wild-type virus and all of the recombinants have identical surface glycoproteins necessary for attachment and penetration into brain cells. Injection of 10^6 PFU into the brain should result in infection and death of a significant number of the brain cells. Death after intracerebral inoculation results from viral replication, spread from cell to cell, and cell destruction before the immune system has a chance to act. Titrations of brain tissue suspended in minimal essential medium containing Eagle's salts and 10% fetal bovine serum showed that the brains of animals inoculated with the viruses that failed to make ICP34.5 contained very little virus. Thus, for the R3616 and R4009 viruses, the recovery was 120 and 100 PFU per gram of brain tissue, respectively. Given the amount of virus in the inoculum (highest concentration tested), it is not clear whether the small amounts of recovered virus represent a surviving fraction of the inoculum or newly replicated virus. In contrast, the amounts of virus recovered from mice inoculated with HSV-1(F)R and R4003 were 6×10^6 , respectively. These results indicate that the failure of the two recombinant viruses to cause death must be related to poor spread of virus in neuronal tissue as a consequence of the inability of mutant viruses to replicate in the CNS, reflecting a reduction in their host range.

In the course of studies designed to determine the function of the $\gamma_{134.5}$ gene product, it was discovered that infection of cells of neuronal origin with mutants incapable of expressing the $\gamma_{134.5}$ gene resulted in shutoff 5 of cellular protein synthesis, whereas infection of cells of non neuronal origin with wild type or mutant viruses resulted in sustained protein synthesis and production of infectious progeny.

10

**EXAMPLE 1 - IMPACT OF $\gamma_{134.5}$
EXPRESSION ON PROGRAMMED CELL DEATH**

Materials and Methods

Cells Vero cells originally obtained from ATCC were propagated in DME media containing 5% calf serum. The human SK-N-SH neuroblastoma (NB) cell line was obtained 15 from ATCC (HTB11) and propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Viruses The isolation of herpes simplex virus 1 strain F, [HSV-1(F)] has been described by Ejercito et al. (J. Gen. Virol., 2: 357-364 (1968) (incorporated herein by reference). The construction of recombinant viruses R3616, R4009, and HSV-1(F) was reported by Chou et al. (Science, 250: 1262-1266, November 30, 1990) (incorporated herein by reference). As illustrated in Fig. 5, R3616 contains a 1 Kbp deletion in both copies of the $\gamma_{134.5}$ gene. In the 20 recombinant R4009 a stop codon was inserted in both copies of the $\gamma_{134.5}$ gene. The $\gamma_{134.5}$ genes in the recombinant R3616 were restored to yield the recombinant HSV-1(F)R.

Virus Infection Cells were generally exposed to the viruses for 2 h at 37°C at multiplicity of infection of 5 30 and then removed and replaced with the 199v media containing 1% calf serum. The infection continued at 37°C for a length of time as indicated for each experiment.

Cells were then either labeled for de novo protein synthesis or analysis of viral DNA and RNA.

³⁵S-methionine labeling At the indicated time post infection, 50uCi of ³⁵S-methionine (specific activity >1,000 Ci/mmol, Amersham Co., Downers Grove, IL) was added to 1ml of 199v media lacking methionine to cells in 6 well dishes. Labeling was continued for 1.5 hr, at which time cells were harvested. Preparation of cell extracts; separation of proteins by electrophoresis in denaturing polyacrylamide gels crosslinked with N,N' Diallyltartardiamide (Bio-Rad Laboratories, Richmond, CA); transfer of polypeptides to nitrocellulose sheets; autoradiography and immunoblot with antibodies have been described by Ackermann et al., J. Virol., 52: 108-118 (1984) (incorporated herein by reference).

Results

HSV-1 recombinant viruses lacking the γ ,34.5 gene induce the shut off protein synthesis in neuroblastoma cells. In the course of screening human cell lines derived from CNS tissues it was apparent that the SK-N SH neuroblastoma cell lines produced 100 fold less mutant viruses than the fully permissive Vero cells. It was also noted, as shown in Fig. 6, that the SK-N-SH neuroblastoma cells infected with R3616 or with R4009 exhibited reduced protein synthesis in cells harvested at 7 hours (left panel) and ceased to incorporate ³⁵S-methionine by 13 hours (right panel) post infection. The phenomenon was observed in SK-N-SH neuroblastoma cells only, and could be attributed specifically to the mutations in the γ ,34.5 gene inasmuch as restoration of the deleted sequences yielded a virus [HSV-1(F)R] which expressed viral proteins at 13 hour post infection (Fig. 6, right panel) and exhibited the parental, wild type phenotype.

The shut off of protein synthesis occurred after the expression of α genes. Viral genes form three major groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion. See Roizman and Sears, 5 Fields' Virology, 2 ed., Fields et al., Eds, 1795-1841 (1990). The α genes do not require de novo protein synthesis for their expression, the β genes which are required for the synthesis of viral DNA require prior synthesis of functional α and β proteins and the onset of 10 viral DNA synthesis. To determine the point at which expression of viral gene functions was terminated in SK-N-SH neuroblastoma cells infected with mutant viruses, infected cell lysates electrophoretically separated in denaturing polyacrylamide gels were transferred to a 15 nitrocellulose sheet and probed with antibody to an α (α 27), a β (viral thymidine kinase) and two abundant γ proteins. Roller and Roizman (J. Virol., 65: 5873-5879 1991) have shown that the latter were the products of U_{26.5} and of U₁₁ genes whose expression at optimal levels 20 requires viral DNA synthesis. As shown in Fig. 7, the SK-N-SH neuroblastoma cells infected with the mutant viruses made normal amounts of α 27 protein (left panel), reduced amounts of the thymidine kinase (β) protein (middle panel), but no detectable γ proteins (left and right panels). In 25 contrast, both the wild type and mutant viruses could not be differentiated with respect to their capacity to replicate or to direct the synthesis of their proteins in Vero cells (Fig. 7).

30 The signal for shut off of protein synthesis is linked to viral DNA synthesis. These experiments were designed to determine whether the shutoff of protein synthesis was linked to a gene whose expression was dependent on viral DNA synthesis. The results of a key experiment are shown in Fig. 8. Replicate SK-N-SH and Vero cell cultures were 35 infected with HSV-1(F) and recombinant viruses and maintained in the presence or absence of inhibitory

concentrations of phosphonoacetate, a drug which blocks viral DNA synthesis. The cells were pulse labeled with S^{35} -methionine at 13 h post infection. The salient feature of the results were that protein synthesis in SK-N-SH cells infected with either R3616 or R4009 was sustained for at least 13 h in the presence of Phosphonoacetate but not in its absence. These results indicated that the signal for cessation of protein synthesis in SK-N-SH neuroblastoma cells infected with mutant viruses was associated with 10 viral DNA synthesis or with a γ gene dependent on viral DNA synthesis for its expression.

Human neuroblastoma cells infected with the γ ,34.5-mutants synthesized viral DNA and accumulated late mRNA even though the shut off of protein synthesis precluded 15 accumulation of late proteins. The evidence presented above indicated that in SK-N-SH neuroblastoma cells an event associated with viral DNA synthesis triggered the shut off of protein synthesis and that the late (γ) viral proteins did not accumulate. We expected, therefore, 20 little or no accumulation of viral DNA and in the absence of viral DNA synthesis, little or no accumulation of late (γ) viral transcripts whose synthesis is dependent on viral DNA synthesis. To our surprise, the amounts of viral DNA recovered from SK-N-SH neuroblastoma cells 17 hours 25 post infection with mutant viruses were comparable to those obtained from wild type parent or repaired [(HSV-1)F]R viruses (Fig. 9, left panel). Furthermore, while the SK-N-SH neuroblastoma cells did not synthesize demonstrable amounts of U,11 protein, the amounts of U,11 gene 30 transcripts which accumulated in cells infected with mutant and wild type viruses were of similar magnitude (Fig. 9 right panel).

The significance of these results stems from three observations. First, in infected cells, protein synthesis 35 reflects a regulatory cascade; a protein synthesis is

replaced by β and later by γ protein synthesis. In all cell lines other than the SK-N-SH neuroblastoma cells infected with the $\gamma_{134.5}$ mutants and tested to date, a block in the synthesis of one group of proteins does not lead to
5 a cessation of total protein synthesis. For example, in cells treated with inhibitors of DNA synthesis like PAA, a subclass of γ proteins dependent for their synthesis on viral DNA synthesis is not made. However, in these cells, β protein synthesis continues beyond the time of their
10 synthesis in untreated infected cells. The striking observations made in the studies on SK-N-SH cells infected with the $\gamma_{134.5}$ null mutants are that (i) all protein synthesis ceased completely, (ii) viral DNA was made and (iii) little, γ mRNA exemplified by U₁₁ mRNA was made even
15 though protein synthesis ceased. These manifestations for viral replication have not been reported previously and are not characteristic of cells infected with wild type virus or any mutant virus infection of cells (e.g. Vero, HEp-2, baby hamster kidney, 143tk- and rabbit skin cell lines and
20 human embryonic lung cells strain) other than those described in this report.

Second, the function of the $\gamma_{134.5}$ gene is to overcome this block in protein synthesis in SK-N-SH cells since repair of the mutation restores the wild type phenotype.

25 Lastly, while the association of cessation of protein synthesis with the onset of viral DNA replication does not exclude the possibility that a product made after infection is responsible of the shut off, the data does support the hypothesis that the cessation of protein synthesis is
30 specifically caused by a known viral gene product interacting with the protein synthesizing machinery of the cell. For example, it has been well established that the product of the HSV-1 gene designated a vhs can shut off cellular protein synthesis after infection. vhs is a
35 structural protein of the virus and is introduced into

cells during infection. It destabilizes mRNA early in infection and its effects are not dependent on viral gene products made after infection. In the experiments set forth above, protein synthesis of wild type and mutant viruses could not be differentiated at 13 hours post infection in cells treated with Phosphonoacetate and hence the phenotype of mutant viruses cannot be attributed to the vhs gene product. This conclusion is reinforced by the observation that viral protein synthesis in SK-N-SH cells was not affected by increasing the multiplicity of infection with wild type virus to values as high as 100 pfu/cell (data not shown). A more likely source for the genetic information for the cessation of protein synthesis is the cell itself.

It has been reported that deprivation of growth factors from cells of neuronal origins results in programmed cell death, which manifests itself initially by the cessation of protein synthesis and subsequently by fragmentation of DNA. Apoptosis in lymphocytes is manifested by degradation of DNA. In the case of other herpes viruses, it has been shown that in B lymphocytes infected with the Epstein-Barr virus, the product of the viral LMP-1 gene induces the host gene Bcl-2 which precludes programmed lymphocyte death (Henderson et al, Cell 65: 1107-1991. Thus, it is apparent that the onset of viral DNA synthesis in neuronal cells triggers programmed cell death by cessation of protein synthesis and that HSV-1 $\gamma_134.5$ gene precludes this response.

The evolution of a HSV-1 gene which would preclude a response to a neuronal stress is not surprising. Infection of neurons, especially sensory neurons, is an essential feature of viral reproductive lifestyle which enables the HSV-1 to remain latent and to survive in human populations. If, as we propose, the function of $\gamma_134.5$ gene is to preclude cell death, the target of the gene would be

neurons rather than lymphocytes since HSV normally infects nerve cells.

The γ ,34.5 gene has several unusual features. The gene lacks a conventional TATAA box or response elements frequently associated with TATAA-less transcriptional units. The sequence which enables the expression of the gene is 12 bp long but repeated as many as 3 times in the wild type strain used in this laboratory. Various assays reported elsewhere indicate that the amounts of gene products produced in cells of non neuronal derivation are smaller than those expressed by most viral genes and that the amounts of the protein made in the absence of viral DNA synthesis were smaller than those made in its presence. The gene is predicted to encode a protein of 263 amino acids. It contains the triplet Ala-Thr-Pro repeated 10 time and accumulates in the cytoplasm. A recent note indicates that 63 amino acid residues near the carboxyl terminus of the γ ,34.5 protein shares 83% identity with a mouse protein MyD116 found in a myeloid leukemic cell line induced to differentiate by interleukin 6 (Lord et al., Nucleic Acid Res. 18: 2823, 1990). The function of MyD116 is unknown. The results presented above demonstrate that the product of the γ ,34.5 gene, the protein ICP34.5, quite clearly enables sustained protein synthesis in SK-N-SH neuroblastoma cells, and it is clear that the gene's expression is sufficient to preclude apoptosis.

The promoter-regulatory elements essential for the expression of γ ,34.5 are contained within three elements of the α sequence, i.e. the direct repeats DR2 and DR4 and the unique U_b sequences. Gel retardation assays failed to show binding of the product of the α 4 gene encoding the major regulatory protein of the virus to any of the elements regulating expression of the γ ,34.5 gene. In transient expression assays, the product of the α 4 or of α 0 genes failed to transactivate a chimeric reporter gene consisting

of the coding sequences of the thymidine kinase gene fused to the 5' non-coding sequences of the $\gamma_{134.5}$ gene. The reporter gene was induced, but to a relatively low level by co-transfection with plasmids containing both $\alpha 4$ and $\alpha 0$ genes. The plasmid encoding the $\alpha 27$ gene had no effect on the expression of the chimeric reporter gene transfected alone although it reduced the induction of the chimeric gene by plasmids containing the $\alpha 0$ and $\alpha 4$ genes.

EXAMPLE 2 - TREATMENT OF PROGRAMMED CELL
10 DEATH (APOPTOSIS) WITH GENE THERAPY

In this example, $\gamma_{134.5}$ gene therapy directed toward the prevention or treatment of apoptosis is described. For the purposes of this example mutated HSV-1 virus is proposed as a vector for introduction of the gene into 15 neuronal cells undergoing or about to undergo programmed cell death. It is also envisioned that this embodiment of the present invention could be practiced using alternative viral or phage vectors, including retroviral vectors and vaccinia viruses whose genome has been manipulated in 20 alternative ways so as to render the virus non-pathogenic. The methods for creating such a viral mutation are set forth in detail in U.S. Patent No. 4,769,331, incorporated herein by reference. Furthermore, it is also envisioned that this embodiment of the present invention could be 25 practiced using any gene whose expression is beneficial in gene therapy, and use of the non-HSV viruses would allow gene therapy in non-neural systems.

Herpes simplex virus has a natural tropism for human CNS tissue. Under wild type conditions, the virus is 30 capable of replicating and multiplying in the nervous system and is neurovirulent. The virus can also establish latent infection in the neurons and can be occasionally reactivated. To establish a vector system for delivery of genes into neurons, the proposed construct of an HSV vector 35 must satisfy the following criteria: 1. Such a vector

should have a natural tropism for CNS and brain tissue. 2. Such a vector should be non-pathogenic; that is, totally avirulent and not reactivatable to cause an infection. 3. Such a vector should consist of constitutive expression of 5 γ ,34.5 to prevent cell death in cells undergoing neurodegeneration. 4. Such a vector so proposed in 3, is suitable for additional foreign gene insertion for gene therapy.

Material and Methods

10 A HSV vector with a mutational lesion in the α 4 gene is constructed. The proposed virus will no longer be able to replicate, multiply and reactivate from latent infection in the CNS. The virus can, in the absence of α 4 gene, establish a latent infection in the neuron. This virus can 15 be obtained by co-transfection of viral DNA with plasmid containing a α 4 expressing cell line. α 4 expressing cell lines and the virus have been reported previously. DeLuca et al., J. Virol., 56: 558-570 (1985).

20 Additionally, such an HSV vector with γ ,34.5 gene under a constitutive expression promoter is also envisioned. This constitutive expression promoter can be the HSV LAT promoter, the LTR promoter of retrovirus or any other foreign promoter specific for neural gene expression. Such 25 a viral vector properly introduced is suitable for prevention of cell death in neuronal cells undergoing apoptosis.

Moreover, such an HSV vector with foreign genes inserted at a neutral location on the viral genome is suitable for delivery of foreign genes into target neurons 30 and for CNS gene therapy. The procedures to generate the above recombinant viruses are those published by Post and Roizman (Cell, 25: 227, 1991) incorporated herein by reference. See also U.S. Patent No. 4,769,331, incorporated herein by reference.

In instances where use of the mutated HSV-1 virus is appropriate the virus can be combined with a pharmaceutically acceptable carrier such as buffered saline and injected at the site of peripheral nerve endings whose axons originate from neural cell bodies undergoing or about to undergo apoptosis. As will be recognized by those skilled in the medical arts the amount of virus administered will vary depending upon several factors, including the vector's ability to target the cells requiring treatment, the extent to which the gene is expressed in the target tissue, and the activity of the expressed protein, among others. An inoculum containing approximately 10^4 - 10^5 viruses in phosphate buffered saline or skim milk has produced successful results in mice. Virus so injected is taken up into the peripheral nerve endings and is then transported via retrograde axonal transport to the neuronal cell bodies. In instances where such peripheral injection is not useful or appropriate, localized intraspinal or intraventricular injection, or direct microinjection of the virus could be utilized.

An appropriately altered non-HSV virus, one with a genome manipulated in such a way as to render the virus non-pathogenic, could be used in a similar manner. Direct microinjection or peripheral injection for delivery to the cell body via retrograde axonal transport are options for viral delivery. Finally, it should also be noted that a biological functional equivalent gene could be utilized for gene therapy in any vector described in this example.

EXAMPLE 3 - USE OF MULTI-POTENT NEURAL
30 CELL LINES TO DELIVER THE γ ,34.5 GENE TO THE CNS

In addition to the viral vector delivery system to CNS and brain tissue, another vector system has been developed recently using cell lines passaged in vitro and engrafting these cells back to the animal. These procedures involve

taking cells of fetal or postnatal CNS origin,
immortalizing and transforming them in vitro and
transplanting the cells back into the mouse brain. These
cells, after engraftment, follow the migration pattern and
5 environmental cue of normal brain cell development and
differentiate in a nontumorigenic, cytoarchitecturally
appropriate manner. This work has been exemplified in
several articles notably Snyder et al., Cell, 68: 33-51,
1992 and Ranfranz et al., Cell, 66: 713-729, 1991.
10 Utilizing appropriately modified techniques, it is possible
to introduce the $\gamma_{134.5}$ gene alone or in combination with
other genes of interest into the cells and engraft. Such a
procedure allows the delivery of the genes to its natural
site. Proper expression of the $\gamma_{134.5}$ gene in these neurons
15 should result in prevention of cell death in
neurodegeneration and preserving cells carrying foreign
genes suitable for gene therapy.

Materials and Methods

20 **Propagation of Cerebellar Cell Lines** Cerebellar cell
lines are generated as described by Ryder et al. (J.
Neurobiol. 21: 356-375, 1990). Lines are grown in
Dulbecco's modified Eagle's medium supplemented with 10%
fetal calf serum (Gibco), 5% horse serum (Gibco), and 2mM
25 glutamine on poly-L-lysine (PLL) (Sigma) (10 μ g/ml)-coated
tissue culture dishes (Corning). The lines are maintained
in a standard humidified, 37°C, 5% CO₂-air incubator and are
either fed weekly with one-half conditioned medium from
confluent cultures and one-half fresh medium or split (1:10
or 1:20) weekly or semiweekly into fresh medium.

30 **Transduction of Cerebellar Progenitor Lines with $\gamma_{134.5}$ Gene**

A recent 1:10 split of the cell line of interest is plated
onto 60 mm tissue culture plates. Between 24 and 48 hr
after plating, the cells are incubated with the

replication-incompetent retroviral vector BAG containing the -myc gene (10^6 - 10^7 colony-forming units [cfu]/ml) plus 8 μ g/ml polybrene for 1-4 hr for introduction of the γ 134.5 gene alone or in combination with other suitable genes for 5 gene therapy, along with the neomycin G418 marker. Cells are then cultured in fresh feeding medium for approximately 3 days until they appear to have undergone at least two doublings. The cultures are then trypsinized and seeded at low density (50-5000 cells on a 100 mm tissue culture 10 dish). After approximately 2 weeks well-separated colonies are isolated by brief exposure to trypsin within plastic cloning cylinders. Colonies are plated in 24-well PLL-coated CoStar plates. At confluence, these cultures are passed to 60 mm tissue culture dishes and expended. A 15 representative dish from each subclone is stained directly in the culture dish using X-gel histochemistry (see Price et al., 1967; Cepko, 1989a, 1989b). The percentage of blue cells is determined under the microscope. Subclones with the highest percentage of blue cells (ideally >90%; at 20 least >50%) are maintained, characterized, and used for transplantation.

Tests for Virus Transmission The presence of helper virus is assayed by measurement of reverse transcriptase activity in supernatants of cell lines as described by 25 Goff et al. (1981) and by testing the ability of supernatants to infect NIH 3T3 cells and generate G418-resistant colonies of X-gal⁺ colonies (detailed in Cepko, 1989a, 1989b). All cerebellar cell lines used for transplantation are helper virus-free as judged by these 30 methods.

Coculture of Neural Cell Lines with Primary Cerebellar Tissue Primary dissociated cultures of neonatal mouse cerebellum are prepared as in Ryder et al. (1990) and seeded at a density of 2×10^6 to 4×10^6 cells per PLL-coated eight-chamber Lab Tek glass or plastic slide 35

(Miles). After the cells settled (usually 24 hr), 10% of a nearly confluent 10 cm dish of the neural cell line of interest is seeded, following trypsinization, onto the slide. The coculture is re-fed every other day and grown 5 in a 50% CO₂-air, humified incubator until 8 or 14 days of coculture.

Preparation of Cells Lines for Transplantation Cells from a nearly confluent but still actively growing dish of donor cells are washed twice with phosphate-buffered saline 10 (PBS), trypsinized, gently triturated with a wide-bore pipette in serum-containing medium (to inactivate the trypsin), gently pelleted (1100 rp for 1 min in a clinical centrifuge), and resuspended in 5 ml of PBS. Washing by pelleting and resuspension of fresh PBS is repeated twice, 15 with the cells finally resuspended in a reduced volume of PBS to yield a high cellular concentration (at least 1 x 10⁶ cells per μ l). Trypan blue (0.05% w/v) is added to localize the inoculum. The suspension is kept well triturated, albeit gently, and maintained on ice prior to 20 transplantation to minimize clumping.

Injections into Postnatal Cerebellum Newborn CD-1 or CF-1 mice are cryoanesthetized, and the cerebellum is localized by transillumination of the head. Cells are administered either via a Hamilton 10 μ l syringe with a beveled 33-gauge needle or a drawn glass micropipette with 25 a 0.75 mm inner diameter and 1.0 mm outer diameter generated from borosilicate capillary tubing (FHC, Brunswick, ME) by a Flaming Brown Micropipette Puller (Model p-87, Sutter Instruments) using the following parameters: heat 750, pull 0, velocity 60, time 0. Best 30 results are achieved with the glass micropipette. The tip is inserted through the skin and skull into each hemisphere and vermis of the cerebellum where the cellular suspension was injected (usually 1-2 μ l per injection). Typically, 35 the following situation should exist: 1 x 10⁷ cells per ml

of suspension; $\times 10^6$ to 2×10^6 cells per injection; one injection in each cerebellar hemisphere and in the vermis. Importantly, the cellular suspension, maintained on ice throughout, is gently triturated prior to each injection in 5 order to diminish clumping and to keep cells suspended.

The injection of BAG virus was performed as described for the cell suspension. The BAG virus stock (8×10^7 G418-resistant cfu/ml) contained, in addition to trypan blue, polybrene at 8 $\mu\text{g}/\text{ml}$.

10 EXAMPLE 4 - TREATMENT OF PROGRAMMED CELL DEATH
 (APOPTOSIS) WITH ICP34.5

As an alternative to the gene therapy methods described for exemplary purposes in Examples 2 and 3, neuronal cells undergoing or about to undergo programmed 15 cell death can also be treated with the protein expressed by the $\gamma_{134.5}$ gene, i.e. ICP34.5. Alternatively, a biological functional equivalent protein could be used in such treatment.

For example, ICP34.5 is isolated from cells expressing 20 the protein and purified using conventional chromatography purification and immunoaffinity purification methods described by Ackerman et al. (*J. Virol.* 58: 843-850, 1986, incorporated herein by reference). The purified protein is next combined with a pharmaceutically appropriate carrier, 25 such as buffered saline or purified distilled water. For administration, the pharmaceutical composition can be injected in one of several ways, as appropriate: (i) intraspinal injection; (ii) intraventricular injection; (iii) direct injection into the area containing the neurons 30 undergoing or about to undergo programmed cell death or any other appropriate method of administration understood by those skilled in the art. Such treatment would be particularly appropriate in the surgical repair of severed peripheral nerves, and the use of proteins as therapeutic

agents is well within the current level of skill in the medical arts in light of the present specification.

**EXAMPLE 5 - ASSAYS FOR CANDIDATE SUBSTANCES FOR
PREVENTION OF PROGRAMMED CELL DEATH (APOPTOSIS)**

5 The γ ,34.5 gene of herpes simplex virus enables the virus to replicate, multiply and spread in the central nervous system and the brain so that the virus is neurovirulent to the host. Recombinant virus lacking the gene lost this ability to penetrate the CNS of the host and 10 become totally avirulent. In examining the nature of this avirulent phenotype in culture, the mutant virus lacking the gene exhibited a total translation shutoff phenotype characteristic of programmed cell death. This mechanism of programmed cell death afforded by the host cell greatly 15 reduced the ability of the virus to multiply and spread. The function of γ ,34.5 in the virus therefore is to inactivate the programmed death of the cell (anti-apoptosis) thereby restoring translation and enabling the virus to replicate to full potential in the host.

20 This anti-apoptotic effect of γ ,34.5 was further examined and its ability to protect neural cells from other environmental stresses which lead to apoptosis was discovered. These environmental stresses include UV, nerve grown factor deprivation and neuronal cell differentiation. 25 This Example describes the use of the γ ,34.5 gene and its protective function to screen for pharmaceutical agents and drugs that mimic the *in vivo* function of γ ,34.5 to prevent neurodegeneration. Such a screening procedure constitutes construction of cell lines expressing γ ,34.5 and a null cell 30 line without the gene and measurement of the viability of the cells after stress treatment by induction of a reporter gene. This can be a host gene promoter tagged by a fluorescence indicator or any other easily assayable marker to signal viability.

Materials and Methods

A test neuroblastoma cell line is established constitutively expressing $\gamma_{134.5}$ and containing a fluorescence tagged (e.g., the α sequence promoter fused to lacZ) cellular gene, or any tag that provides the easily assayable marker to signal viability. A neuroblastoma null cell line consisting of α -lacZ indicator gene and the same host indicator gene is also established, along with a Vero cell line consisting of α -lacZ indicator gene and the same host indicator gene. Environmental stresses are then applied that normally would (1) trigger the α sequence promoter activation; (2) trigger the protection afforded by $\gamma_{134.5}$ as signaled by viability after stress treatment; and (3) trigger cell programmed death in the absence of $\gamma_{134.5}$. Candidate substances of pharmaceutically appropriate drugs and agents can be tested in such an assay. The proposed scheme of the assay for scoring of positive candidates is shown in outline form in Table 2.

TABLE 2
EXPERIMENTAL FLOW CHART: ASSAY FOR CANDIDATE
SUBSTANCE ABLE TO PREVENT PROGRAMMED CELL DEATH

	<u>CELL LINES</u>	<u>ACTION</u>	<u>EXPECTATION</u>
20	A. neuroblastoma cells constitutively express $\gamma_{134.5}$ and a second inducible promoter-indicator gene	stress followed by induction of second promoter	viability as measured by induction of a reporter gene hours after stress
25	B. neuroblastoma cells expressing α -lacZ and a second inducible promoter	stress followed by induction of second promoter	1. apoptosis related stress: α -lacZ induced; second promoter not induced 2. toxicity: no induction of α -lacZ and the second inducible promoter
30			
35			

- C. vero cells,
 expressing α -lacZ
 and a second inducible
 promoter gene

stress followed
 by induction

 1. α -lacZ not induced
 2. toxicity factor excluded, determined from expression of inducible second promoter

10 EXAMPLE 6 - ASSAY FOR CANDIDATE SUBSTANCES FOR
ACTIVATION OF PROGRAMMED CELL DEATH
(APOPTOSIS) FOR TREATMENT OF CANCER OR
TUMOROGENIC DISEASES AND FOR SUPPRESSION OF HSV INFECTION

In order to induce cell death in tumor cells, it is
desirable to block the expression of the anti-apoptosis
gene or the activity of the protein expressed by the gene.
As such, it is desirable to develop procedures that will
allow screening for candidate substances which trigger cell
death in tumor cells. In addition, since expression of the
₂₀ γ 34.5 gene of HSV-1 has been shown to prevent apoptosis in
neuronal cells so that the virus can replicate, multiply
and spread in the CNS (that is, so that the virus can
become neurovirulent), a substance capable of blocking
 γ 34.5 expression or inhibiting the action of ICP34.5 can be
₂₅ expected to suppress HSV neurovirulence (or the virulence of
other viruses relying on a similar mechanism) by allowing
apoptosis to occur in infected neurons.

It has been found that the protection afforded by γ 34.5 can be extended to protect other cells from environmental stresses, and indeed the gene has a generalized anti-apoptotic effect. The promoter for the gene γ 34.5 lies in the α sequence of HSV and, at time of stress, the promoter is activated. It can be assumed that the α sequence promoter contains apoptosis responsive elements and cellular factors (transcription factors in particular) that mediate the expression of anti-apoptosis gene are apoptotic in nature. These cellular factors are therefore the targets of the present assay to screen for drugs or agents that would inactivate their ability to

induce the anti-apoptosis gene. The assay involves the use of the α sequence promoter and its inducibility by conditions which induce apoptosis as an indicator assay which screens for therapeutic agents and drugs capable of blocking the expression of the anti-apoptosis gene and therefore allow the cell to die of programmed cell death.

A test plasmid construct bearing the α sequence and coding sequence up to the 28th amino acid of $\gamma_134.5$ is fused to the lacZ reporter gene or any other readily assayable reporter gene. The construct is introduced into a neuroblastoma or PC12 cell line by G418 selection and a clonal and continuous cell line for screening purposes is established. A control plasmid construct bearing an HSV late promoter, a promoter which would normally not be expressed in cell lines and which further would not be induced to express by apoptosis-inducing stress is fused to the same indicator gene. This construct is also introduced into a continuous clonal cell line and serves as a control for the test cell line. Environmental stresses that trigger the α sequence promoter activation and that cause programmed cell death are then defined. These conditions include UV injury, virus infection, nerve growth factor deprivation, and the influence of antibodies on cell surface receptors, among others. Candidate substances or pharmaceutically appropriate drugs and agents are then tested in assays for their ability to block the α sequence promoter activation at time of stress.

The assay of the present invention allows the screening and identification of pharmaceutically appropriate drugs and agents targeted at various cellular factors that induce the expression of anti-apoptosis gene. By inactivating essential cellular factors, these agents should be able to allow cell programmed death to occur. Such positive candidates would then be appropriately administered (via intravenous, intrathecal, or direct injection, or via oral administration) in order to induce programmed cell death in tumor cells, in neurons infected

with the herpes virus, or in cells infected with a virus the virulence of which is dependent upon an anti-apoptotic effect. The use of proteins and other chemotherapeutic substances in antitumor therapy is well known in the art, 5 and therefore, it is considered that the use and dosages of candidate substances for treatment of tumorogenic diseases (e.g., cancer) or herpes infection is well within the skill of the present state of the medical arts in light of the present specification. See U.S. Pat. Nos. 4,457,916; 10 4,529,594; 4,447,355; and 4,477,245, all incorporated herein by reference. These positive candidates can also be used to identify intermediates in the pathways leading to cell programmed death.

Materials and Methods

15 W5 cell lines are established in 96 well culture dishes coated with collagen. Control cell lines containing the promoter fusion element are also established in such 96 well dishes. The test candidate substances are added to the medium in individual wells containing both the test and 20 control cell lines set up in 96 well dishes. The cells are then briefly exposed to UV or other stresses. 8 hr post stress induction, cells are washed with PBS-A twice, and fixed with 0.5 ml containing 2% (v/v) formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature. The 25 cells are rinsed again with PBS and then stained with 2ml 5mM potassium ferrocyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, and 1mg/ml X-gal (diluted from a 40mg/ml stock solution in dimethyl sulfoxide) in PBS. Cells expressing β-Galactosidase were stained blue after incubation at 37°C 30 for 2-3 days.

Results

The construct described above with the lacZ reporter gene was introduced into PC12 cell line. A new cell line W5 was clonally established by G418 selection. The W5 cell 35 line was then tested for activation of the α sequence promoter under suboptimal conditions names in 3 above. The results are: (a) The above cells, when exposed briefly to

UV for 2 minutes, turn blue upon staining the fixed cells with X-gal at 6-10 hr post UV exposure. (b) The above cells, when exposed to HSV-1(F) virus at multiplicity of infection of 5, also turn blue upon staining at 8 hr post infection. (c) The above cells turn light blue when nerve growth factor (rat, 7S) is introduced into the medium to allow differentiation processes. (d) The cells turn darker blue when Nerve Growth Factor is removed from the medium after differentiation is complete and the cells have become dependent on nerve growth factor for survival. (e) Little or no difference in color development is seen in cells starved for serum (0% fetal bovine serum) and those fully supplied in 10% fetal bovine serum. (f) The above experiments are repeated with control promoter fusion elements to control for the true inhibition of anti-apoptosis gene expression rather than toxicity-induced cell death. By this procedure, the positive candidates that can induce cell death in cells will therefore render the following phenotypes: (i) Introduction of stress to the test cell line in the absence of this substance will give rise to blue colored cells. (ii) Introduction of stress to the test cell line in the presence of same substance will give rise to white cells.

(iii) Introduction of stress to control cell lines with our without this putative substance will have no effect on the color of cells.

The present invention has been disclosed in terms of specific embodiments which are believed by the inventors to be the best modes for carrying out the invention. However, in light of the disclosure hereby provided, those of skill in the various arts will recognize that modifications can be made without departing from the intended scope of the invention. The exemplary embodiments set forth herein and all other modifications and embodiments are intended to be within the scope and spirit of the present invention and the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Roizman, Bernard
Chou, Joany
- (ii) TITLE OF INVENTION: Methods and Compositions For Gene Therapy, Tumor Therapy, Viral Infection Therapy and Prevention of Programmed Cell Death (Apoptosis)
- (iii) NUMBER OF SEQUENCES: 35
- (iv) CORRESPONDENCE ADDRESS:
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 - (C) CITY: Chicago
 - (D) STATE: Illinois
 - (E) COUNTRY: USA
 - (F) ZIP: 60610
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/861,233
 - (B) FILING DATE: 31-MAR-1992
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 133 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTTAAAGTCGGCGGG

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 133 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCAGCCCCGGC CCCCCGGCGGC CGAGACGAGC GAGTTAGACA GGCAAGCACT ACTCGCCTCT	60
GCACGCCACAT GCTTGCTGT CAAACTCTAC CACCCCGGCA CGCTCTCTGT CTCCATGGCC	120
CGCCGCCCGCC GCC	133

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 291 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCGCGGCCCGGCGCCCGGGCCCGGGCCACGGG CGCCGTCCCC ACCGCACAGT	60
CCCAGGTAAC CTCCACGCCAAC AACTCGGAAC CCGCGGTCAG GAGCGCGCCC GCGGCCGCC	120
CGCCGCCCGCC CCCCGCCAGT GGGCCCCCGC CTTCTTGTTC GCTGCTGCTG CGCCAGTGGC	180
TCCACGTTCC CGAGTCCGCG TCCGACGACG ACGATGACGA CGACTGGCCG GACAGCCCCC	240
CGCCCGAGCC GGCGCCAGAG GCCCGGCCCA CCGCCGCCGC CCCCCGCC	291

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 595 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACCGCCCGGC	GCGGGCCCGG	GGGGCGGGGC	TAACCCCTCC	CACCCCCCT	CACGCCCTT	60
CCGCCTTCCG	CCGCGCCTCG	CCCTCCGCCT	GCGCGTCACC	GCAGAGCACC	TGGCGCGCCT	120
GCGCCTGCGA	CGCGCGGGCG	GGGAGGGGGC	GCCGGAGCCC	CCCGCGACCC	CCGCGACCCC	180
CGCGACCCCC	GCGACCCCCG	CGACCCCCGC	GACCCCCGCG	ACCCCCGCGA	CCCCCGCGAC	240
CCCCCGCGACC	CCCGCGCGGG	TGCGCTTCTC	GCCCCACGTC	CGGGTGCGCC	ACCTGGTGGT	300
CTGGGCCTCG	GCCGCCCCGC	TGGCGCGCCG	CGGCTCGTGG	GCCCGCGAGC	GGGCGACCG	360
GGCTCGGTTC	CGGCGCCGGG	TGGCGGAGGC	CGAGGCGGTC	ATCGGGCCGT	GCCTGGGCC	420
CGAGGCCCCGT	GCCCAGGGCCC	TGGCCCGCGG	AGCCGGCCCG	GCGAACTCGG	TCTAACGTTA	480
CACCCGAGGC	GGCCTGGGTC	TTCCGCGGAG	CTCCCGGGAG	CTCCGACCCA	AGCCGCTCTC	540
CGGAGAGACG	ATGGCAGGAG	CCGCGCATAT	ATACGCTGGG	AGCCGGCCCG	CCCCC	595

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGGCAGGGCC CGCCCTCGGA GGGCGGGACT GGCCAATCGG CGGCCGCCAG CGCAGCGGGGG	60
CCCGGCCAAC CAGCGTCCGC CGAGTCTTCG GGGCCCGGCC CACTGGCGG GAGTTACCGC	120
CCAGTGGGCC GGGCCGCCCA CTTCCCGGTA TGGTAATTAA AAACTTACAA GAGGCCTTGT	180
TCCGCTTCCC GGTATGGTAA TTAGAAACTC ATTAATGGC GGCCCCGGCC GCCCTTCCCG	240
CTTCCGGCAA TTCCCGCGGC CCTTAATGGG CAACCCCGGT ATTCCCCGCC T	291

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 150 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTAAAGCGG TGGCGGGCGGG CAGCCCGGGC CCCCCGCCGA GACTAGCGAG TTAGACAGGC	60
AAGCACTACT CGCCTCTGCA CGCACATGCT TGCCTGTCAA ACTCTACCAC CCCGGCACGC	120
TCTCTGTCTC CATGGCCCCGC CGCCGCCGCC	150

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 503 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCGCGGGCCC CCGCCGCCCG CGGCCGCCCCG GGCCCACGGG CGCCGTCCCA ACCGCACAGT	60
CCCAGGTAAC CTCCACGCCA AACTCGGAAC CCGCGGTCAAG GAGCGCGCCC GCGGCCGCC	120
CGCCGCCGCC CCCCCGCCGGT GGGCCCCCGC CTTCTTGTTC GCTGCTGCTG CGCCAGTGGC	180
TCCACGTTCC CGAGTCCGCG TCCGACGACG ACGATGACGA CGACTGGCCG GACAGCCCCC	240
CGCCCCAGTC GGCGCCAGAG GCCCCGCCCA CCGCCGCCGC CCCCCGCCCG CCGGGCCCCC	300
ACCGCCCGGC GTGGGCCCCGG GGGGCGGGGC TGACCCCTCC CACCCCCCTT CGCGCCCCCTT	360
CCGCCTTCCG CCGCGCCTCG CCCTCCGCCT GCGCGTCACC GCGGAGCACC TGGCGCGCCT	420
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGGAGCCC CCCGCGACCC CCGCGACCCC	480
CGCGACCCCCC GCGACCCCCG CGA	503

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 368 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCCCGCGAC CCCCCGCGCGG GTGCGCTTCT CGCCCCACGT CCGGGTGCAC CACCTGGTGG	60
TCTGGGCCTC GGCGGCCCGC CTGGCGCGCC GCGGCTCGTG GGCCCGCGAG CGGGCCGACC	120
GGGCTCGGTT CCGGCGCCGG GTGGCGGAGG CCGAGGGCGGT CATCGGGCCG TGCCTGGGC	180
CCGAGGGCCCG TGCCCCGGCC CTGGCCCGCG GAGCCGGCCC GGCGAACTCG GTCTAACGTT	240
ACACCCGAGG CGGCCTGGGT CTTCCGCGGA GCTCCGGGA GCTCCGCACC AAGCCGCTCT	300
CCGGAGAGAC GATGGCAGGA GCCGGCGATA TATACGCTTG GAGCCAGCCC GCCCTCACAG	360
GGCGGGCC	368

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 187 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGCAGGGACT GGCCAATCGG CGGCCGCCAG CGCGGCGGGG CCCGGCCAAC CAGCGTCCGC	60
CGAGTCTTCG GGGCCCGGCC CATTGGGCGG GAGTTACCGC CCAATGGGCC GGGCCGCCA	120
CTTCCCGGTA TGGTAATTAA AAACTTGCAA GAGGCCTTGT TCCGCTTCCC GGTATGGTAA	180
TTAGAAACTC ATTAATGGGC GGCCCCGGCC GCCCTTCCCG CTTCCGGCAA TTCCCGCGGC	240
CCTTAATGGG CAACCCCGGT ATTCCCCGCC T	271

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTAAAGTCA C

11

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 256 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCGGGCGGGC AGCCCCCCCCG CGGCCGAGAC TAGCGAGTTA GACAGGCAAG CACTACTCGC	60
CTCTGCACGC ACATGCTTGC CTGTCAAAC TCTACCACCCC GGCACGCTCT CTGTCTCCAT	120
GGCCCGCCGC CGCCGCCGCC ATCGCGGCC CGGCCGCC CGGCCGCCCG GGCCCACGGG	180
CGCGGTCCCC ACCGCACAGT CCCAGGTAAC CTCCACGCC AACTCGGAAC CGGTGGTCAG	240
GAGCGCGCCC GCGGCC	256

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 154 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTGGGGCCC CGCCTTCTTG TTCGCTGCTG CTGCGCCAGT GGCTCCACGT TCCCGAGTCC	60
GCGTCCGACG ACGACGATGA CGACGACTGG CGGGACAGCC CCCCGCCCGA GCCGGCGCCA	120
GAGGCCCGGC CCACCGCCGC CGCCCCCCGC CCCC	154

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 212 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACCGCCCCGGC GCGGGCCCGG GGGGCAGGGC TAACCCCTCC CACCCCCCT CACGCCCTT	60
CCGCCTTCCG CGCGCCCTCG CCCTCCGCCT GCGCGTCACC GCGGAGCACC TGGCGCGCCT	120
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGAAGCCC CCCGCGACCC CCGCGACCCC	180
CGCGACCCCCC GCGACCCCCG CGACCCCCGC GA	212

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 356 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCCCCGCGAC CCCCGCGCGG GTGCGCTTCT CGCCCCACGT CCGGGTGCAC CACCTGGTGG	60
TCTGGGCCTC GGCGCGCCGC CTGGCGCGCC GCAGCTCGTG GGCGCGCGAG CGGGCCGACC	120
GGGCTCGGTT CGGGCGCCGG GTGGCGGAGG CCGAGGGCGGT CATCGGGCCG TGCCTGGGGC	180
CCGAGGCCCG TGCCCGGGCC CTGGCGCGCG GAGCCGGCCC GGCGAACTCG GTCTAACGTT	240
ACACCCGAGG CGGCCTGGGT CTTCCGCGGA GCTCCCGGGA GCTCCACACC AAGCCGCTCT	300
CCGGAGAGAC GATGGCAGGA GCCGCGCATA TATACGCTGG GAGCCGGCCC GCCCCC	356

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

64

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGGCGGGCC CGCCCTCGGA ,GGGCGGGACT GGCCAATCGG CGGCCGCCAG CGCGGCAGGG	60
CCCAGGCAAC CAGCGTCCGC CGAGTCGTG GGGCCCGGCC CACTGGCGG TAACTCCGC	120
CCAGTGGGCC GGGCCGCCA CTTCCCGTA TGGTAATTAA AAACTTGCAA GAGGCCTTGT	180
TCCGCTTCCC GGTATGGTAA TTAGAAACTC ATTAATGGC GGCCCCGGCC GCCCTTCCCG	240
CTTCCGGCAA TTCCCGCGGC CCTTAATGGG CAACCCGGT ATTCCCCGCC T	291

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTTAAAGCGC

10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 431 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCGGGCGGGC AGCCCCCCC CGGCCGAGAC TAGCGAGTTA GACAGGCAAG CACTACTCGC	60
CTCTGCACGC ACATGCTTGC CTGTCAAAC CTACCACCCC GGCACGCTCT CTGTCTCCAT	120
GGCCCGCCGC CGCCGCCGCC ATCGCGGCC CCGCCGCC CGGCCGCCGG GGCCCACGGG	180

CGCGGTCCCC ACCGCACAGT CCCAGGTAAC CTCCACGCC AACTCGGAAC CCGTGGTCAG	240
GAGCGCGCCC CGGGCCGCC CGCCGCGCC CCCCAGCGGT GGGCCCCCGC CTTCTTGTTC	300
GCTGCTGCTG CGGCAGTGGC TCCAGGTTCC GGAGTCCGCG TCCGACGACG ACGATGACGA	360
CGACTGGCCG GACAGCCCCC CGCCCGAGCC GGCGCCAGAG GCCCGGCCA CCGCCGCCGC	420
CCCCCCGC C	431

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 212 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACCGCCCCGGC GCAGGGCCAG GGGGCGGGGC TGACCCCTCC CACCCCCCT CACGCCCTT	60
CCGCCTTCCG CCGCGCCTCG CCCTCCGCCT GCGCGTCACC GCAGAGCACC TGGCGCGCCT	120
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGGAGCCC CCCGCGACCC CGCGACCC	180
CGCGACCCCCC GCGACCCCCG CGACCCCCGC GA	212

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 356 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

66

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCCCCGCGAC	CCCCGGCGGG	'GTGCGCTTCT	CGCCCCACGT	CCGGGTGCGC	CACCTGGTGG	60
TCTGGGCCTC	GGCCGGCCCGC	CTGGCGCGCC	GCGGCTCGTG	GGCCCGCGAG	CGGGCCGACC	120
GGGCTCGGTT	CCGGCGCCGG	GTGGCGGAGG	CCGAGGCCGT	CATCGGGCCG	TGCCTGGGCC	180
CCAAGGCCCG	CGCCCGGGCC	CTGGCCCGCG	GAGCCGGCCC	GGCGAACTCG	GTCTAACGTT	240
ACACCCGAGG	CGGCCTGGGT	CTTCCGCGGA	GCTCCCGGGA	GCTCCACACC	AAGCCGCTCT	300
CCGGAGAGAC	GATGGCAGGA	GCCGCGATA	TATACGCTGG	GAGCCGGCCC	GCCCCC	356

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 207 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAGGCGGGCC	CGCCCTCGGA	GGGCAGGACT	GGCCAATCGG	CGGCCGCCAG	CGCGGCGGGG	60
CCCAGCCAAC	CAGCGTCCGC	CGAGTCGTG	GGGCCCCGCC	CACTGGCGG	TAACTCCCGC	120
CCAGTGGGCC	GGGCCGCCCA	CTTCCCGGTA	TGGTAATTAA	AAACTTGCAA	GAGGCCTTGT	180
TCCGCTTCCC	GGTATGGTAA	TTAGAAACTC	ATTAATGGC	GGCCCCGGCC	GCCCTTCCCG	240
CTTCCGGCAA	TTCCCGCGGC	CCTTAATGGG	CAACCCCGGT	ATTCCCCGCC	T	291

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTAACCTAGA CTAGTCTAGC

20

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCTGATCA GATCGCATTG

20

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCCGGACATG GAACGAGTAC GACGACGCCAG CCGACGCCGC CGGCGACCGG GCCCCGGG

58

68

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTGCTCATGC TGCTGCGTCG GCTGCGGCCG CCGCTGGCCC GGGGCCCCGTA C

51

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Ala Arg Arg Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 258 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

His Arg Gly Pro Arg Arg Pro Pro Arg Pro Pro Gly Pro Thr Gly Ala Val
 1 5' 10 15

Pro Thr Ala Gln Ser Gln Val Thr Ser Thr Pro Asn Ser Glu Pro Ala
 20 25 30

Val Arg Ser Ala Pro Ala Ala Ala Pro Pro Pro Pro Ala Ser Gly
 35 40 45

Pro Pro Pro Ser Cys Ser Leu Leu Leu Arg Gln Trp Leu His Val Pro
 50 55 60

Ala Glu Ser Ala Ser Asp Asp Asp Asp Asp Asp Asp Trp Pro Asp Ser
 65 70 75 80

Pro Pro Pro Glu Pro Ala Pro Glu Ala Arg Pro Thr Ala Ala Ala Pro
 85 90 95

Arg Pro Arg Ser Pro Pro Pro Gly Ala Gly Pro Gly Gly Gly Ala Asn
 100 105 110

Pro Ser His Pro Pro Ser Arg Pro Phe Arg Leu Pro Pro Arg Leu Ala
 115 120 125

Leu Arg Leu Arg Val Thr Ala Glu His Leu Ala Arg Leu Arg Leu Arg
 130 135 140

Arg Ala Gly Gly Glu Gly Ala Pro Glu Pro Pro Ala Thr Pro Ala Thr
 145 150 155 160

Pro Ala Thr Pro
 165 170 175

Ala Thr Pro Ala Thr Pro Ala Thr Pro Ala Arg Val Arg Phe Ser Pro
 180 185 190

His Val Arg Val Arg His Leu Val Val Trp Ala Ser Ala Ala Arg Leu
 195 200 205

Ala Arg Arg Gly Ser Trp Ala Arg Glu Arg Ala Asp Arg Ala Arg Phe
 210 215 220

Arg Arg Arg Val Ala Glu Ala Glu Ala Val Ile Gly Pro Cys Leu Gly
 225 230 235 240

Pro Glu Ala Arg Ala Arg Ala Leu Ala Arg Gly Ala Gly Pro Ala Asn
 245 250 255

Ser Val

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Ala Arg Arg Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

His Arg Gly Pro Arg Arg Pro Pro Arg Pro Pro Gly Pro Thr Gly Ala Val
1 5 10 15

Pro Thr Ala Gln Ser Gln Val Thr Ser Thr Pro Asn Ser Glu Pro Ala
20 25 30

Val Arg Ser Ala Pro Ala Ala Pro Pro Pro Pro Pro Ala Gly Gly
35 40 45

Pro Pro Pro Ser Cys Ser Leu Leu Leu Arg Gln Trp Leu His Val Pro
50 55 60

Glu Ser Ala Ser Asp Asp Asp Asp Asp Asp Trp Pro Asp Ser Pro
65 70 75 80

Pro Pro Glu Ser Ala Pro Glu Ala Arg Pro Thr Ala Ala Ala Pro Arg
85 90 95

71

Pro Pro Gly Pro His Arg Pro Ala Trp Ala Arg Gly Ala Gly Leu Thr
 100 105 110

Pro Pro Thr Pro Pro Arg Ala Pro Ser Ala Phe Arg Arg Ala Ser Pro
 115 120 125

Ser Ala Cys Ala Ser Pro Arg Ser Thr Trp Arg Ala Cys Ala Cys Asp
 130 135 140

Ala Arg Ala Gly Arg Gly Arg Arg Ser Pro Pro Arg Pro Pro Arg Pro
 145 150 155 160

Pro Arg Pro Pro Arg Pro Pro Arg Pro
 165

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 180 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Pro Arg Gly Cys Ala Ser Arg Pro Thr Ser Gly Cys Ala Thr Trp Trp
 1 5 10 15

Ser Gly Pro Arg Pro Pro Ala Trp Arg Ala Ala Ala Arg Gly Pro Ala
 20 25 30

Ser Gly Pro Thr Gly Leu Gly Ser Gly Ala Gly Trp Arg Arg Pro Arg
 35 40 45

Arg Ser Ser Gly Arg Ala Trp Gly Pro Arg Pro Val Pro Gly Pro Trp
 50 55 60

Pro Ala Glu Pro Ala Arg Arg Thr Arg Ser Asn Val Thr Pro Glu Ala
 65 70 75 80

Ala Trp Val Phe Arg Gly Ala Pro Gly Ser Ser Ala Pro Ser Arg Ser
 85 90 95

Pro Glu Arg Arg Trp Gln Glu Pro Arg Ile Tyr Thr Leu Gly Ala Ser
 100 105 110

72

Pro Pro Ser Gln Gly Gly Pro Pro Arg Gly Arg Asp Trp Pro Ile Gly
 115 120 125

Gly Arg Gln Arg Gly Gly Ala Arg Pro Thr Ser Val Arg Arg Val Phe
 130 135 140

Gly Ala Arg Pro Ile Gly Arg Glu Leu Pro Pro Asn Gly Pro Gly Arg
 145 150 155 160

Pro Leu Pro Gly Met Val Ile Lys Asn Leu Gln Glu Ala Leu Phe Arg
 165 170 175

Phe Pro Val Trp
 180

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Met Ala Arg Arg Arg Arg Arg His Arg Gly Pro Arg Arg Pro Arg Pro
 1 5 10 15

Pro Gly Pro Thr Gly Ala Val Pro Thr Ala Gln Ser Gln Val Thr Ser
 20 25 30

Thr Pro Asn Ser Glu Pro Val Val Arg Ser Ala Pro Ala Ala
 35 40 45

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 126 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Gly Gly Pro Pro Pro Ser Cys Ser Leu Leu Leu Arg Gln Trp Leu His
1 5 10 15

Val Pro Glu Ser Ala Ser Asp Asp Asp Asp Asp Asp Asp Trp Pro Asp
20 25 30

Ser Pro Pro Pro Glu Pro Ala Pro Glu Ala Arg Pro Thr Ala Ala Ala
35 40 45

Pro Arg Pro Arg Ser Pro Pro Pro Gly Ala Gly Pro Gly Gly Ala
50 55 60

Asn Pro Ser His Pro Pro Ser Arg Pro Phe Arg Leu Pro Pro Arg Leu
65 70 75 80

Ala Leu Arg Leu Arg Val Thr Ala Glu His Leu Ala Arg Leu Arg Leu
85 90 95

Arg Arg Ala Gly Gly Glu Gly Ala Pro Lys Pro Pro Ala Thr Pro Ala
100 105 110

Thr Pro Ala Thr Pro Ala Thr Pro Ala Thr Pro Ala Thr Pro
115 120 125

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Ala Arg Val Arg Phe Ser Pro His Val Arg Val Arg His Leu Val Val
1 5 10 15

Trp Ala Ser Ala Ala Arg Leu Ala Arg Arg Gly Ser Trp Ala Arg Glu
20 25 30

74

Arg Ala Asp Arg Ala Arg Phe Arg Arg Arg Val Ala Glu Ala Glu Ala
 35 40 45

Val Ile Gly Pro Cys Leu Gly Pro Glu Ala Arg Ala Arg Ala Leu Ala
 50 55 60

Arg Gly Ala Gly Pro Ala Asn Ser Val
 65 70

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 179 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Ala Arg Arg Arg Arg Arg His Arg Gly Pro Arg Arg Pro Arg Pro
 1 5 10 15

Pro Gly Pro Thr Gly Ala Val Pro Thr Ala Gln Ser Gln Val Thr Ser
 20 25 30

Thr Pro Asn Ser Glu Pro Val Val Arg Ser Ala Pro Ala Ala Ala Pro
 35 40 45

Pro Pro Pro Pro Ala Gly Gly Pro Pro Pro Ser Cys Ser Leu Leu Leu
 50 55 60

Arg Gln Trp Leu Gln Val Pro Glu Ser Ala Ser Asp Asp Asp Asp Asp
 65 70 75 80

Asp Asp Trp Pro Asp Ser Pro Pro Pro Glu Pro Ala Pro Glu Ala Arg
 85 90 95

Pro Thr Ala Ala Ala Pro Arg Pro Arg Ser Pro Pro Pro Gly Ala Gly
 100 105 110

Pro Gly Gly Gly Ala Asp Pro Ser His Pro Pro Ser Arg Pro Phe Arg
 115 120 125

75

Leu Pro Pro Arg Leu Ala Leu Arg. Leu Arg Val Thr Ala Glu His Leu
130 135 140

Ala Arg Leu Arg Leu Arg Arg Ala Gly Gly Glu Gly Ala Pro Glu Pro
145 150 155 160

Pro Ala Thr Pro
165 170 175

Ala Thr Pro

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Ala Arg Val Arg Phe Ser Pro His Val Arg Val Arg His Leu Val Val
1 5 10 15

Trp Ala Ser Ala Ala Arg Leu Ala Arg Arg Gly Ser Trp Ala Arg Glu
20 25 30

Arg Ala Asp Arg Ala Arg Phe Arg Arg Arg Val Ala Glu Ala Glu Ala
35 40 45

Val Ile Gly Pro Cys Leu Gly Lys Glu Ala Arg Ala Arg Ala Leu Ala
50 55 60

Arg Gly Ala Gly Pro Ala Asn Ser Val
65 70

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Asp Glu Tyr Asp Asp Ala Ala Asp Ala Ala Gly Asp Arg Ala Pro
1 5 10 15

Gly Met

WHAT IS CLAIMED IS:

1. A method of preventing or treating programmed cell death in neuronal cells, the method comprising:
5 (a) preparing a non-pathogenic vector comprising the γ 34.5 gene; and
 (b) introducing the non-pathogenic vector into neuronal cells undergoing or likely to undergo programmed cell death.

10 2. The method of claim 1 wherein the vector comprises an HSV-1 or HSV-2 virus altered in such a way as to render it non-pathogenic.

15 3. The method of claim 2 wherein said alteration comprises deletion of the ICP4 gene, the α 4 gene, or the α 0 gene of the HSV-1 or HSV-2 viral genome.

20 4. The method of claim 2 wherein said alteration comprises a mutational lesion of the ICP4 gene, the α 4 gene, or the α 0 gene of the HSV-1 or HSV-2 viral genome.

25 5. The method of claim 1 wherein said vector comprises a retrovirus altered, a vaccinia virus, a picornavirus, a coronavirus, a eunyavirus, a togavirus, or a rhabdovirus in such a way as to render it non-pathogenic.

30 6. The method of claim 1 wherein the vector comprises a multipotent neural cell line.

25 7. The method of claim 1 further comprising introducing the vector into the neuronal cells undergoing or likely to undergo programmed cell death by a process comprising transplanting cells of the multipotent neural cell line of claim 6 into a region of the central nervous system in which said neuronal cells undergoing or likely to undergo programmed cell death are located.

8. The method of claim 1 further comprising introducing the vector into neuronal cells of an animal by injection of the vector at the site of the peripheral nerve endings of the neuronal cells undergoing or likely to undergo cell death.

9. The method of claim 1 further comprising introducing the vector into neuronal cells in culture likely to undergo or undergoing cell death by incubation of the vector with the neuronal cells.

10 10. A viral vector comprising the HSV-1 or HSV-2 virus having a genomic alteration rendering the viral vector non-pathogenic.

11. The viral vector of claim 10 wherein said genomic alteration comprises deletion of the ICP4 gene, the $\alpha 4$ gene, or the $\alpha 0$ gene of the HSV-1 or HSV-2 genome.

12. The viral vector of claim 10 wherein said genomic alteration comprises a mutational lesion of the ICP4 gene, the $\alpha 4$ gene, or the $\alpha 0$ gene of the HSV-1 or HSV-2 genome.

13. A viral vector comprising a non-pathogenic retrovirus, vaccinia virus, picornavirus, coronavirus, eunyavirus, togavirus, or rhabdovirus.

14. A method of preventing or treating programmed cell death in neuronal cells, the method comprising:

25 (a) preparing ICP34.5 or a biological functional equivalent thereof;

(b) combining the ICP34.5 or the biological functional equivalent with a pharmaceutically acceptable carrier to form a pharmaceutical composition; and

(c) administering the composition to neurons likely to undergo or undergoing programmed cell death.

15. The pharmaceutical composition of claim 14, said pharmaceutical composition comprising ICP34.5 or a
5 biological functional equivalent thereof in a pharmaceutically acceptable carrier.

16. The method of claim 14 wherein ICP34.5 or its biological functional equivalent is prepared by the method comprising:

10 (a) preparing a nucleic acid segment capable of encoding ICP34.5 or a biological functional equivalent; and

(b) expressing the segment to produce the ICP34.5 or biological functional equivalent protein.

15 17. The method of claim 16 wherein the segment is transferred into a host cell and the host cell is cultured under conditions suitable for expression of the segment.

20 18. The method of claim 17 wherein the nucleic acid segment is transferred by transfection or transformation of a recombinant vector into the host cell.

19. The method of claim 17 further comprising isolating and purifying the protein.

25 20. The method of claim 14 wherein the composition is administered to an animal by direct, intrathecal or intravenous injection, or by oral administration.

21. The method of claim 14 wherein the composition is administered to neuronal cells in culture by incubation of

said cells in a medium comprising the composition such that the protein will enter the cells.

22. A method for determining the ability of a candidate substance to protect cells from programmed cell death, said method comprising:

- (a) preparing a neuronal cell line sensitive to programmed cell death;
- (b) combining the cells of said cell line with the candidate protective substance;
- 10 (c) altering the incubation solution such that the cells are exposed to conditions or substances capable of inducing programmed cell death; and
- (d) determining whether the candidate substance has protected the cells from programmed cell death.

15 23. The method of claim 22 wherein the candidate substance is a putative biological functional equivalent of ICP34.5.

24. A method for determining the ability of a candidate substance to potentiate the protective function
20 of ICP34.5 or biological functional equivalents thereof, said method comprising:

- (a) preparing a neuronal cell line sensitive to programmed cell death;
- (b) combining the cells of said cell line with the candidate potentiating substance;
- 25 (c) adding to the incubation medium ICP34.5 or a biological functional equivalent thereof;

(d) altering the incubation solution such that the cells are exposed to conditions or substances capable of inducing programmed cell death; and

5 (e) determining whether the candidate substance has potentiated the protective effects of ICP34.5 or the biological functional equivalent.

10 25. A method for determining the ability of a candidate substance to act as an inhibitor of either γ 34.5 expression or activity of ICP34.5 or biological functional equivalents thereof, said method comprising:

(a) preparing a neuronal cell line sensitive to programmed cell death;

(b) combining the cells of said cell line with the candidate inhibitory substance;

15 (c) adding to the incubation medium ICP34.5 or a biological functional equivalent thereof;

(d) altering the incubation solution such that the cells are exposed to conditions or substances capable of inducing programmed cell death; and

20 (e) determining whether the candidate substance has inhibited the protective effect of ICP34.5 or the biological functional equivalent.

26. A method of delivering a gene for gene therapy, the method comprising:

25 (a) combining the gene for gene therapy with any one of the vectors of claims 10, 11, 12 or 13;

(b) combining the gene and vector with a pharmacologically acceptable carrier to form a pharmaceutical composition; and

5 (c) administering said pharmaceutical composition so that the gene and vector will reach the intended cell targets.

27. The method of claim 26 wherein said pharmaceutical composition is introduced by injection into an animal at the site of said cell targets.

10 28. The method of claim 26 wherein said cell targets are in the central nervous system and the pharmaceutical composition is introduced by injection into an animal at the site of the peripheral nerve ending which originate from neurons located at the site of said cell targets.

15 29. The method of claim 26 wherein said pharmaceutical composition is introduced by intrathecal or intravenous injection.

20 30. The pharmaceutical composition of claim 26 comprising the gene and vector combined with a pharmacologically acceptable carrier.

31. A method of treating tumorogenic diseases or viral infections, the method comprising:

(a) preparing a candidate substance of claim 25;

25 (b) combining the candidate substance with a pharmaceutically acceptable carrier to form a pharmaceutical composition; and

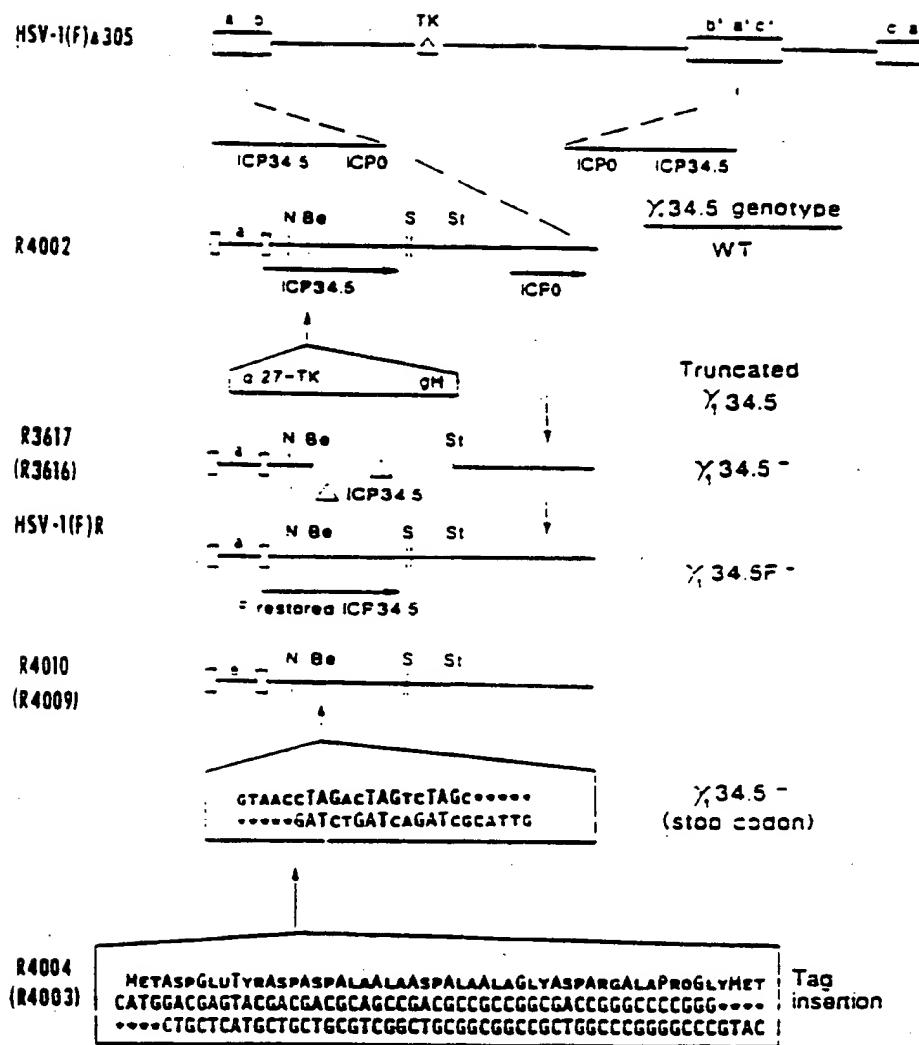
(c) administering the composition to tumor cell targets.

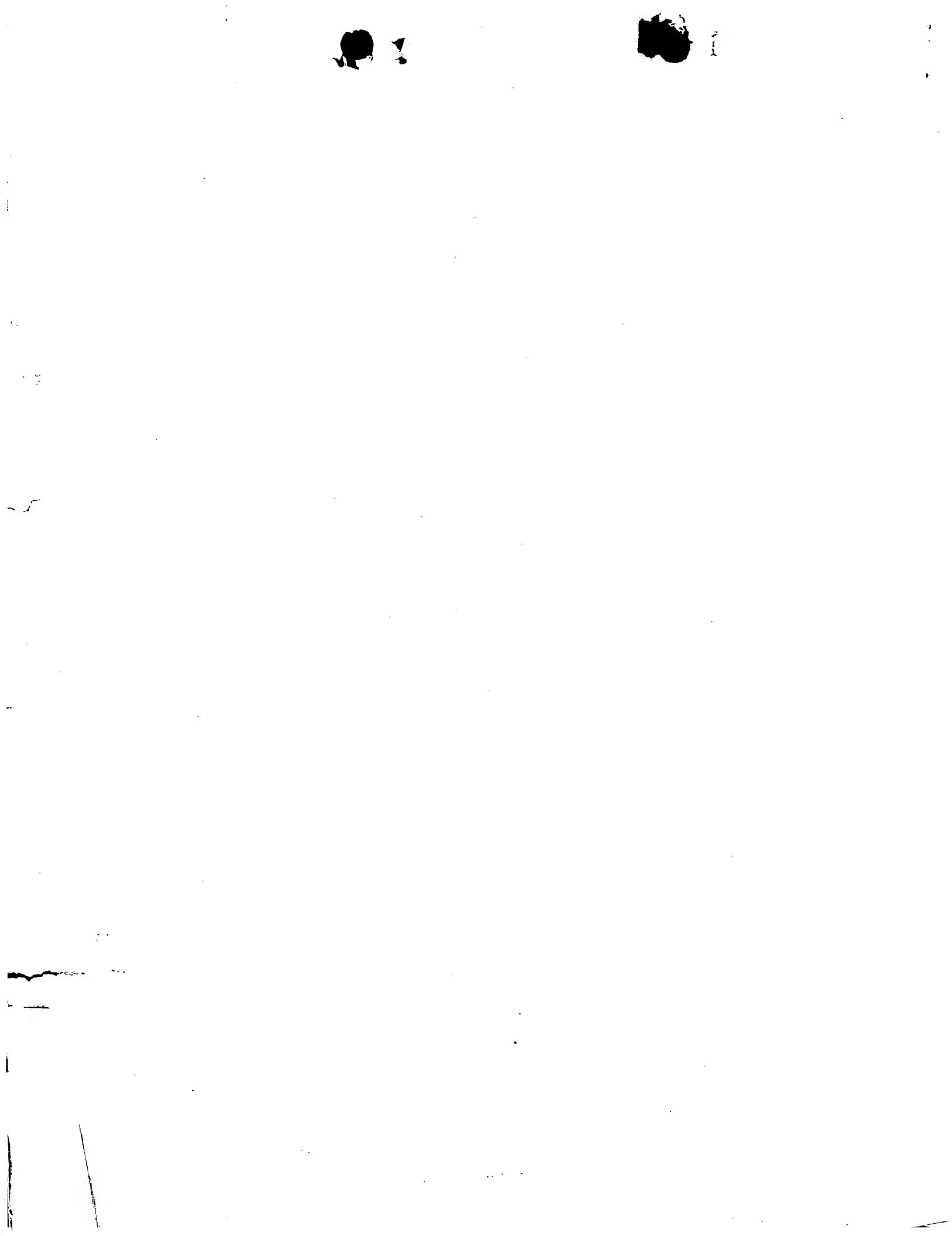
32. The method of claim 31 wherein the pharmaceutical composition is administered by injection directly into the site of the cell targets, by intravenous injection, by intraspinal injection, or orally.

5 33. The pharmaceutical composition of claim 31 comprising the candidate substance combined with a pharmaceutically acceptable carrier.

FIG. 1

Fig. 2

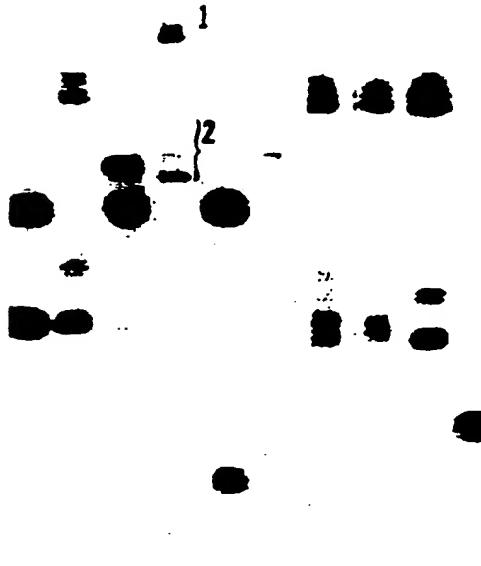




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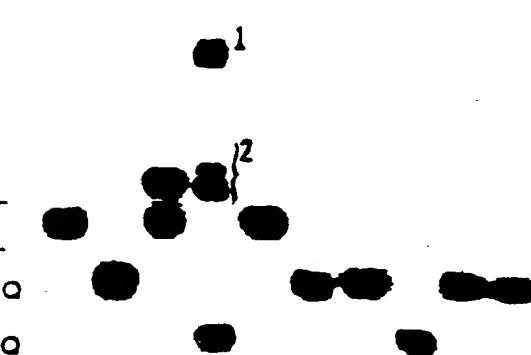
FIG. 3

pRB143
HSV-1(F)
pRB3615
R4002
pRB3616
R3616
HSV-1(F)R
Δ305
R4009
R4009



probe: Nco I to Sph I of ICP34.5 (pRB4081)

pRB143
HSV-1(F)
pRB3615
R4002
pRB3616
R3616
HSV-1(F)R
Δ305
R4009
R4009

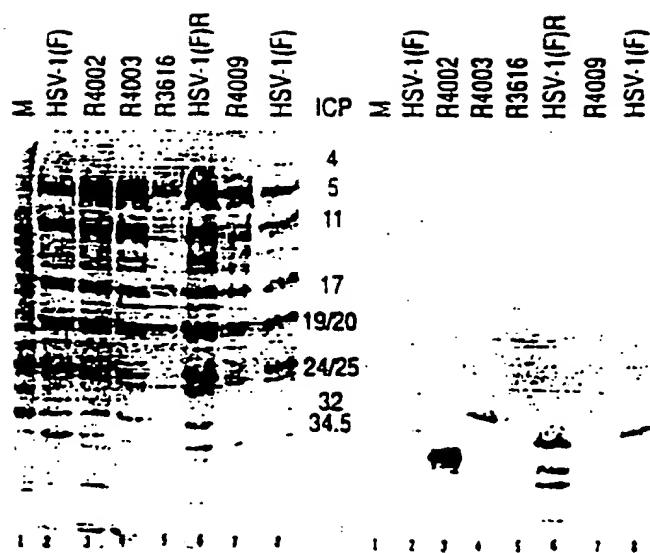


probe: Bam HI Q (pRB103)



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Fig. 4





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FIG. 5

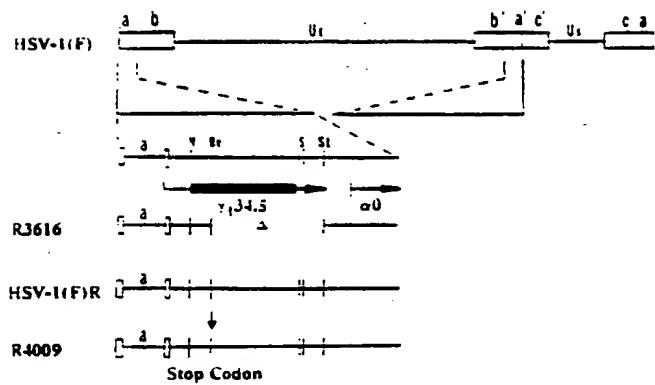


Fig 1. Schematic Diagram



FIG. 6

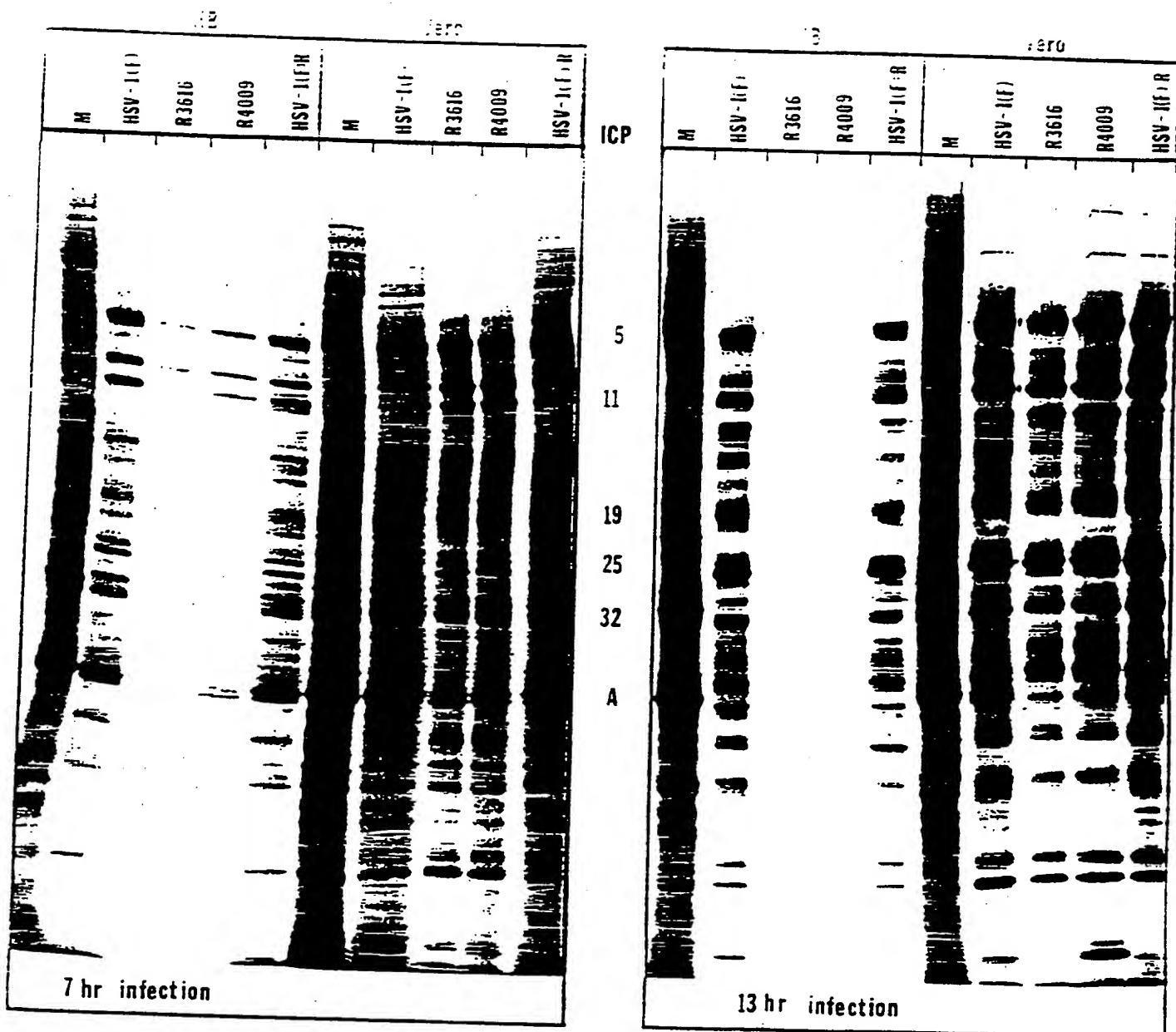




FIG. 7

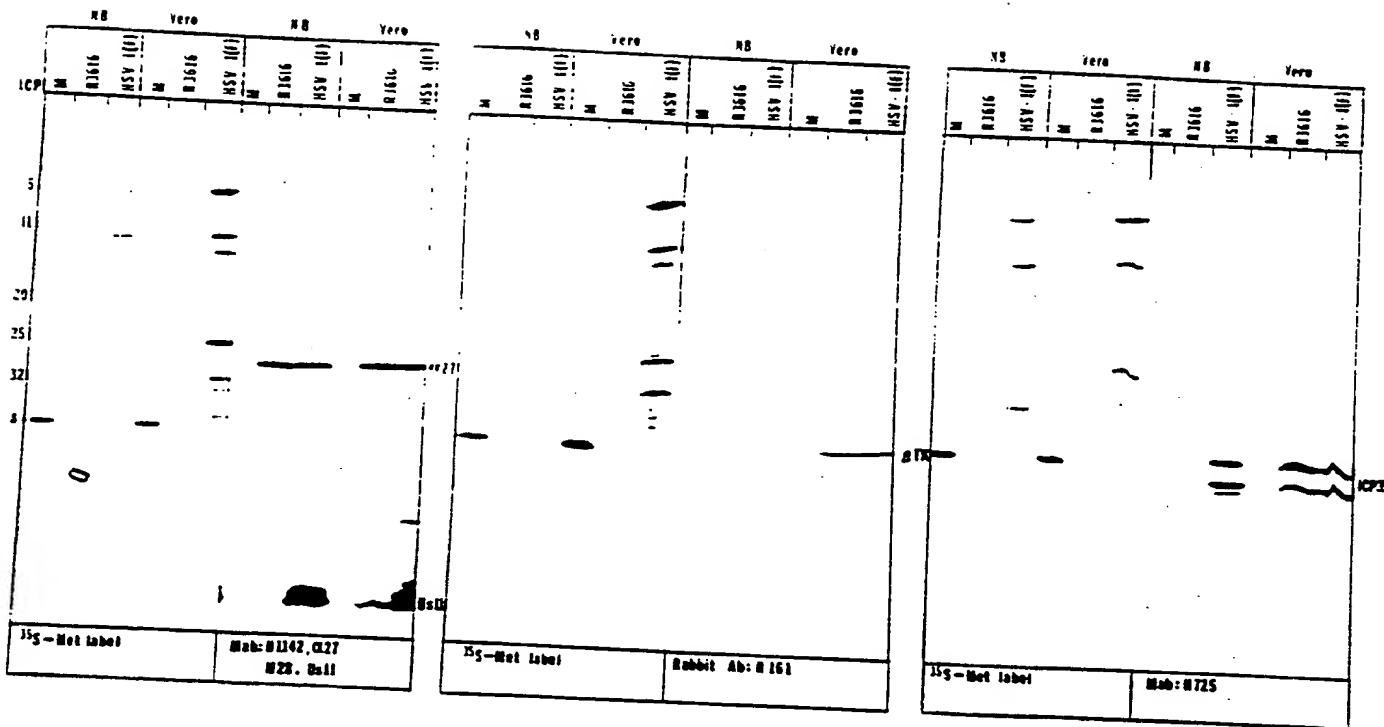


Fig. 7. Glut + Lysine

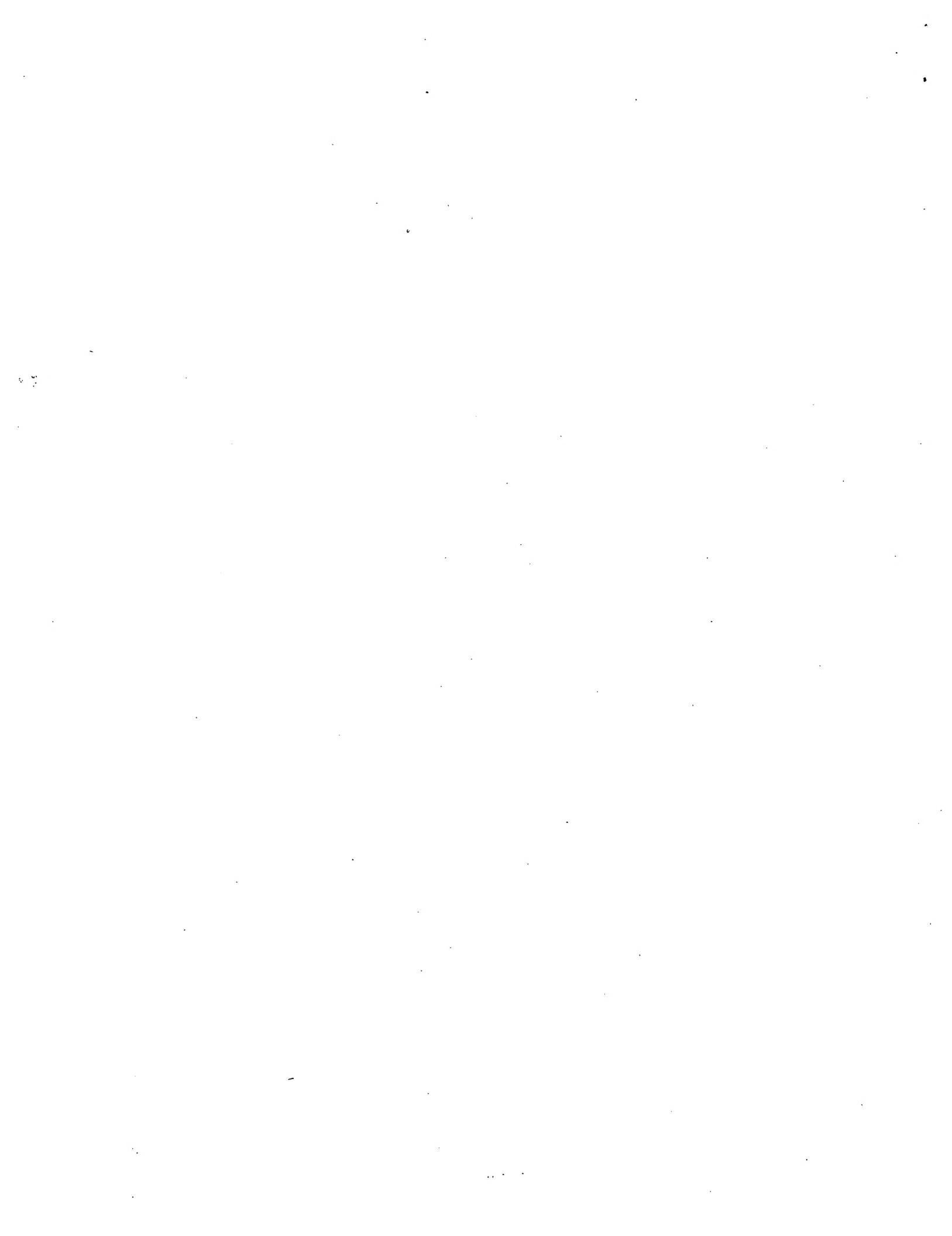


FIG. 8

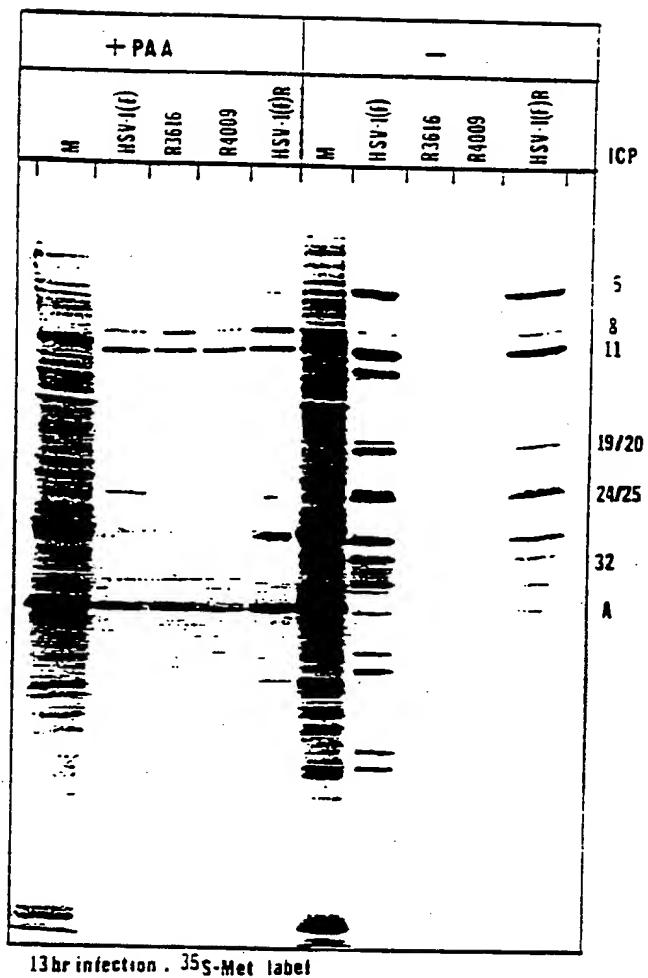
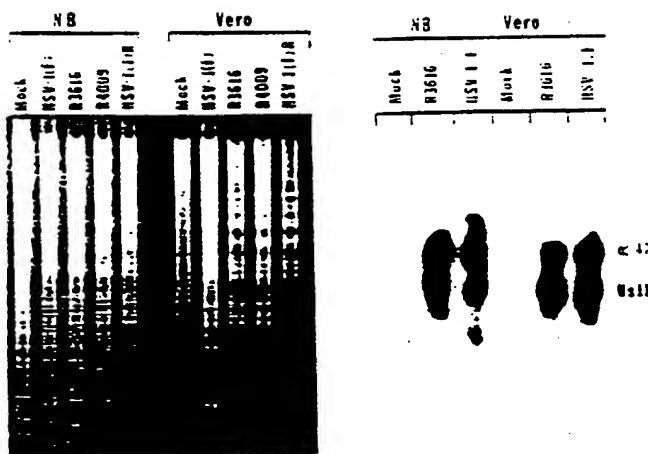


Fig 4. Chou + Roizman

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Fig. 9

Fig. 10. ~~10a + 10b~~

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01801

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01K 63/00; A61K 37/00, 31/70; A01N 37/18; C12N 15/00; C12Q 1/02;
US CL :424/93B; 435/29, 320.1; 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93B; 435/29, 320.1; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Chem. Abs.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 65, issued 15 December 1991, S. Henderson, "Induction of <u>bcl-2</u> Expression by Epstein-Barr Virus Latent Membrane Protein 1 Protects Infected B Cells from Programmed Cell Death", pages 1107-1115, see entire document.	1-33
Y	Cell, Volume 67, issued 29 November 1991, A. Strasser et al, " <u>bcl-2</u> Transgene Inhibits T Cell Death and Perturbs Thymic Self-Censorship", 889-899, see entire document.	1-33

Further documents are listed in the continuation of Box C.

See patent family annex.

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O document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 June 1993

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International application No.

PCT/US93/01801

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Vol. 67, issued 29 November 1991, C.L. Sentman et al, " <u>bcl-2</u> Inhibits Multiple Forms of Apoptosis but Not Negative Selection in Thymocytes", pages 879-888, see entire document.	1-33
Y	Nature, Vol. 349, issued 14 February 1991, C. D. Gregory et al, "Activation of Epstein-Barr Virus Latent Genes Protects Human B Cells From Death by Apoptosis", pages 612-614, see entire document.	1-33



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A01K 63/00, A61K 37/00, 31/70 · A01N 37/18, C12N 15/00 C12Q 1/02	A1	(11) International Publication Number: WO 93/19591 (43) International Publication Date: 14 October 1993 (14.10.93)
(21) International Application Number: PCT/US93/01801		(74) Agents: NORTHRUP, Thomas, E. et al.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).
(22) International Filing Date: 26 February 1993 (26.02.93)		
(30) Priority data: 07/861,233 31 March 1992 (31.03.92) US		(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).
(71) Applicant: ARCH DEVELOPMENT CORPORATION [US/US]; 1101 East 58th Street, The University of Chicago, Chicago, IL 60637 (US).		
(72) Inventors: ROIZMAN, Bernard ; 5555 S. Everett, Chicago, IL 60637 (US). CHOU, Joany ; 5227 S. Kimbark, Chicago, IL 60615 (US).		Published <i>With international search report.</i>

(54) Title: METHODS AND COMPOSITIONS FOR GENE, TUMOR, AND VIRAL INFECTION THERAPY, AND PREVENTION OF PROGRAMMED CELL DEATH (APOPTOSIS)

(57) Abstract

The present invention relates to methods of treatment of programmed cell death (apoptosis) through the use of the HSV-1 gene γ 134.5 or the product of its expression, ICP34.5. The gene and its expression have been demonstrated to be required for HSV-1 neurovirulence, and in particular, to act as an inhibitor of neuronal programmed cell death which allows for viral replication. Use of the gene therapy, or the protein itself, can be expected to result in inhibition of programmed cell death in various neurodegenerative diseases. This invention also relates to novel vectors for gene therapy, including modified herpes virus. Methods are presented for conducting assays for substances capable of mimicing, potentiating or inhibiting the expression of γ 134.5 or the activity of ICP34.5. Also, methods are disclosed for the treatment of tumorigenic diseases, including cancer, and for treatment of herpes and other viral infections using inhibitors of γ 134.5 expression or ICP34.5 activity.

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METHODS AND COMPOSITIONS FOR GENE, TUMOR, AND VIRAL INFECTION THERAPY, AND PREVENTION OF PROGRAMMED CELL DEATH (APOPTOSIS)

BACKGROUND OF THE INVENTION

The government may own certain rights in the present invention pursuant to grants from the National Cancer Institute (CA47451) and from the National Institute for Allergy and Infectious Diseases (AI24009 and AI1588), and the United States Public Health Service.

1. Field of the Invention

The present invention is directed to methods for blocking or delaying programmed cell death, for delivery of gene therapy to specific cells, and for treatment of cancer and other tumorigenic diseases, as well as treatment of viral infections, through the potentiation of programmed cell death in tumor or viral host cells. The present invention is also directed to assays for candidate substances which can either inhibit, or potentiate programmed cell death.

2. Description of the Related Art

a. Programmed Cell Death (Apoptosis)

In the last decade there has been increasing acceptance in the scientific community of the idea that cells may actually be internally programmed to die at a certain point in their life cycle. As an active cellular mechanism programmed cell death, or apoptosis, has several important implications. First, it is clear that such an active process can provide additional means of regulating cell numbers as well as the biological activities of cells. Secondly, mutations or cellular events which potentiate apoptosis may result in premature cell death. Third, a form of cell death which is dependent on a specific active cellular mechanism can at least potentially be suppressed.

Finally, an inhibition of preprogrammed cell death would be expected to lead to aberrant cell survival and could be expected to contribute to oncogenesis.

In general, apoptosis involves distinctive morphological changes including nuclear condensation and degradation of DNA to oligonucleosomal fragments. In certain circumstances it is evident that apoptosis is triggered by or is preceded by changes in protein synthesis. Apoptosis appears to provide a very clean process for cellular destruction, in that the cells are disposed of by specific recognition and phagocytosis prior to bursting. In this manner cells can be removed from a tissue without causing damage to the surrounding cells. Thus, it can be seen that programmed cell death is crucial in a number of physiological processes, including morphological development, clonal selection in the immune system, and normal cell maturation and death in other tissue and organ systems.

It has also been demonstrated that cells can undergo apoptosis in response to environmental information. Examples include the appearance of a stimulus, such as glucocorticoid hormones for immature thymocytes, or the disappearance of a stimulus, such as interleukin-2 withdrawal from mature lymphocytes, or the removal of colony stimulating factors from hemopoietic precursors (for a review of literature see Williams, Cell, 85; 1097-1098, June 28, 1991). Furthermore, it has recently been demonstrated that the response of removal to nerve growth factor from established neuronal cell cultures mimics target removal, or axotomy, or other methods of trophic factor removal, and it has been postulated that the cellular mechanism involved in this response is a triggering of a suicide program or programmed cell death following the nerve growth factor removal. (See Johnson et al., Neurobiol. of Aging, 10: 549-552, 1989). The authors

propose a "death cascade" or "death program", which envisions that trophic factor deprivation initiates the transcription of new mRNA and the subsequent translation of that mRNA into death associated proteins which act in sequence to ultimately produce "killer proteins". Such an intracellular mechanism seems to fit well with the characteristics of apoptosis discussed above, eg., death of specific cells without the release of harmful materials and without the disruption of tissue integrity. Furthermore, the authors indicate that inhibitors of macromolecular synthesis prevented the death of neurons in the absence of nerve growth factor.

Studies have been conducted to explore the possibility that tumor cells could be eliminated by artificially triggering apoptosis. The APO-1 monoclonal antibody can induce apoptosis in several transformed human B and T cell lines. The antibody binds to a surface protein and could act either by mimicking a positive death-inducing signal or by blocking the activity of a factor required for survival. Also, anti-FAS antibodies have similar effects, and the recent cloning and sequencing of the gene for the FAS antigen has shown that it is a 63 kilodalton transmembrane receptor. Itoh et al., Cell 66: 233-243 (1991).

However, it is important to note that neither APO-1 nor FAS can function exclusively as triggers for cell death. Both are cell surface receptors that may activate quite different responses under other circumstances. Moreover, these antigens are not confined to tumor cells and their effect on normal cells is certainly an important consideration, as is the possible appearance of variants that no longer display the antigens.

It has also been demonstrated that the cell death induced by a range of cytotoxic drugs, including several used in cancer therapy, has also been found to be a form of

apoptosis. In fact, the failure of apoptosis in tumor cells could be of fundamental importance in contributing not only to the evasion of physiological controls on cell numbers, but also to resistance both to natural defenses 5 and to clinical therapy.

It has also been demonstrated that expression of the bcl-2 gene can inhibit death by apoptosis. The bcl-2 gene was isolated from the breakpoint of the translocation between chromosomes 14 and 18 found in a high proportion of 10 the most common human lymphomas, that being follicular B cell lymphomas. The translocation brings together the bcl-2 gene and immunoglobulin heavy chain locus, resulting in an aberrantly increased bcl-2 expression in B cells. Subsequently, Henderson et al. (Cell, 65: 1107-1115, 1991) 15 demonstrated that expression of latent membrane protein 1 in cells infected by Epstein-Barr virus protected the infected B cells from programmed cell death by inducing expression of the bcl-2 gene. Sentman et al. (Cell, 67: 879-88, November 29, 1991) demonstrated that expression of 20 the bcl-2 gene can inhibit multiple forms of apoptosis but not negative selection in thymocytes, and Strasser et al. (Cell, 67: 889-899, November 29, 1991) demonstrated that expression of a bcl-2 transgene inhibits T cell death and can perturb thymic self- censorship. Clem et al. (Science, 25 245: 1388-1390, November 29, 1991) identified a specific baculovirus gene product as being responsible for blocking apoptosis in insect cells.

b. Herpes Virus Infections and Neurovirulence

The family of herpes virus includes animal viruses of 30 great clinical interest because they are the causative agents of many diseases. Epstein-Barr virus has been implicated in B cell lymphoma; cytomegalovirus presents the greatest infectious threat to AIDS patients; and Varicella Zoster Virus, is of great concern in certain parts of the

world where chicken pox and shingles are serious health problems. A worldwide increase in the incidence of sexually transmitted herpes simplex (HSV) infection has occurred in the past decade, accompanied by an increase in 5 neonatal herpes. Contact with active ulcerative lesions or asymptomatically excreting patients can result in transmission of the infectious agent. Transmission is by exposure to virus at mucosal surfaces and abraded skin, which permit the entry of virus and the initiation of viral 10 replication in cells of the epidermis and dermis. In addition to clinically apparent lesions, latent infections may persist, in particular in sensory nerve cells. Various stimuli may cause reactivation of the HSV infection. Consequently, this is a difficult infection to eradicate. 15 This scourge has largely gone unchecked due to the inadequacies of treatment modalities.

The known herpes viruses appear to share four significant biological properties:

1. All herpes viruses specify a large array of 20 enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase, etc.), DNA synthesis (e.g., DNA polymerase helicase, primase), and, possibly, processing of proteins (e.g., protein kinase), although the exact array 25 of enzymes may vary somewhat from one herpesvirus to another.

2. Both the synthesis of viral DNAs and the assembly of capsids occur in the nucleus. In the case of some 30 herpes viruses, it has been claimed that the virus may be de-enveloped and re-enveloped as it transits through the cytoplasm. Irrespective of the merits of these conclusions, envelopment of the capsids as it transits through the nuclear membrane is obligatory.

3. Production of infectious progeny virus is invariably accompanied by the irreversible destruction of the infected cell.

4. All herpes viruses examined to date are able to 5 remain latent in their natural hosts. In cells harboring latent virus, viral genomes take the form of closed circular molecules, and only a small subset of viral genes is expressed.

Herpes viruses also vary greatly in their biologic 10 properties. Some have a wide host-cell range, multiply efficiently, and rapidly destroy the cells that they infect (e.g., HSV-1, HSV-2, etc.). Others (e.g., EBV, HHV6) have a narrow host-cell range. The multiplication of some 15 herpes viruses (e.g., HCMV) appears to be slow. While all herpes viruses remain latent in a specific set of cells, the exact cell in which they remain latent varies from one 20 virus to another. For example, whereas latent HSV is recovered from sensory neurons, latent EBV is recovered from B lymphocytes. Herpes viruses differ with respect to the clinical manifestations of diseases they cause.

Herpes simplex viruses 1 and 2 (HSV-1, HSV-2), are among the most common infectious agents encountered by humans (Corey and Spear, N. Eng. J. Med., 314: 686-691, 1986). These viruses cause a broad spectrum of diseases 25 which range from mild and nuisance infections such as recurrent herpes simplex labialis, to severe and life-threatening diseases such as herpes simplex encephalitis (HSE) of older children and adults, or the disseminated infections of neonates. Clinical outcome of herpes 30 infections is dependent upon early diagnosis and prompt initiation of antiviral therapy. However, despite some successful therapy, dermal and epidermal lesions recur, and HSV infections of neonates and infections of the brain are associated with high morbidity and mortality. Earlier

diagnosis than is currently possible would improve therapeutic success. In addition, improved treatments are desperately needed.

Extrinsic assistance has been provided to infected
5 individuals, in particular, in the form of chemicals. For example, chemical inhibition of herpes viral replication has been effected by a variety of nucleoside analogues such as acyclovir, 5-fluorodeoxyuridine (FUDR), 5-iododeoxyuridine, thymine arabinoside, and the like.

10 Some protection has been provided in experimental animal models by polyspecific or monospecific anti-HSV antibodies, HSV-primed lymphocytes, and cloned T cells to specific viral antigens (Corey and Spear, N. Eng. J. Med., 314: 686-691, 1986). However, no satisfactory treatment
15 has been found.

The $\gamma_134.5$ gene of herpes simplex virus maps in the inverted repeat region of the genome flanking the L component of the virus. The discovery and characterization of the gene was reported in several articles (Chou and Roizman, J. Virol., 57: 629-635, 1986, and J. Virol., 64: 1014-1020, 1990; Ackermann et al., J. Virol., 58: 843-850, 1986). The key features are: (i) the gene encodes a protein of 263 amino acid in length; (ii) the protein contains Ala-Thr-Pro trimer repeat ten times in the middle 25 of the coding sequence; (iii) the protein is basic in nature and consists of large number of Arg and Pro amino acids; (iv) the promoter of the gene maps in the a sequence of the genome which also serves several essential viral functions for the virus; (v) the *cis*-acting element 30 essential for the expression of the gene $\gamma_134.5$ is contained within the a sequence, in particular, the DR2 (12 base pair sequence repeated 22 times) and U_b element. This type of promoter structure is unique to this gene and not shared by other viral gene promoters.

5 The function of the gene γ ,34.5 in its ability to enable the virus to replicate, multiply and spread in the central nervous system (CNS) was demonstrated by a set of recombinant viruses and by testing their abilities to cause fatal encephalitis in the mouse brain. The mutant viruses lacking the gene therefore lost their ability to multiply and spread in the CNS and eyes and therefore is non-pathogenic. See Chou et al., Science, 250: 1212-1266, 1990.

10 The γ ,34.5 gene functions by protecting the nerve cells from total protein synthesis shutoff in a manner characteristic of programmed cell death (apoptosis) in neuronal cells. The promoter appears to contain stress response elements and is transactivated by exposure to UV 15 irradiation, viral infection, and growth factor deprivation. These data suggest that the gene γ ,34.5 is transactivated in the nerve cells at times of stress to prevent apoptosis.

20 The significance of these findings therefore lies in the fact that γ ,34.5 extends viability or lends protection to the nerve cells so that in this instance, the virus can replicate and spread from cell to cell -- defined as neurovirulence. It also appears that the protection can be extended to other toxic agents or environmental stresses to 25 which the cell is subjected. An important aspect about the nature of the neurons, unlike any other cells in human, is the fact that neurons in the brain, eyes or CNS do not regenerate which forms the basis of many impaired neurological diseases. Any genes or drugs that extend the life of cells from death or degeneration can be expected to 30 have a significant impact in the area of neural degeneration.

The role of γ ,34.5, and anti-apoptosis factors, in infected cells is in its early stages of elucidation.

Recent studies have suggested that Epstein-Barr virus enhances the survival capacity of infected cells through latent membrane protein 1(LMP1)-induced up-regulation of bcl-2. In that system it is postulated that LMP 1 induced
5 bcl-2 up regulation gives virus infected B cells the potential to by-pass physiological selection and gain direct access to long lived memory B cell pools. However, bcl-2 expression fails to suppress apoptosis in some situations, for example upon withdrawal of interleukin-2 or
10 interleukin-6. Moreover, the intracellular mechanism of action of bcl-2 expression remains unknown.

c. Programmed Cell Death and Disease Therapy

In light of the foregoing, it is apparent that the expression of γ ,34.5 in CNS cells added an extra dimension of protection to the neurons against viral infection, and naturally occurring and stress-induced apoptosis. An appreciation of this extra dimension of protection can be utilized in novel and innovative means for control and treatment of central nervous system (CNS) disorders.
15 Specifically, treatment of CNS degenerative diseases, including Alzheimer's disease, Parkinson's disease, Lou Gerig's disease, and others the etiology of which may be traceable to a form of apoptosis, and the treatment of which is currently very poor, could be improved
20 significantly through the use of either the γ ,34.5 gene in gene therapy or the protein expressed by γ ,34.5 as a therapeutic agent. This is especially critical where the death of neuronal cells is involved, due to the fact that, as noted, such cells do not reproduce post-mitotically.
25 Since a finite number of neurons are available it is crucial to have available methods and agents for their protection and maintenance. γ ,34.5 is also a very useful gene for assays of substances which mimic the effect of γ ,34.5 and block stress of biologically induced programmed
30 cell death.
35

Furthermore, the HSV-1 virus, appropriately modified so as to be made non-pathogenic, can serve as a vehicle for delivery of gene therapy to neurons. The HSV-1 virus is present in neurons of the sensory ganglia of 90% of the world's human population. The virus ascends into neuronal cell bodies via retrograde axonal transport, reaching the axon from the site of infection by the process of neurotropism. Once in the neuronal cell body the virus remains dormant until some form of stress induces viral replication (e.g. UV exposure, infection by a second virus, surgery or axotomy). As noted, the use of HSV-1 as a vector would necessitate construction of deletion mutants to serve as safe, non-pathogenic vectors. Such a virus would act as an excellent vector for neuronal gene therapy and its use would be an especially important development since few methods of gene therapy provide a means for delivery of a gene across the central nervous system's blood-brain barrier.

Moreover, other viruses, such as HSV-2, picornavirus, coronavirus, eunyavirus, togavirus, rhabdovirus, retrovirus or vaccinia virus, are available as vectors for $\gamma_134.5$ gene therapy. As discussed with regard to the use of HSV-1 viruses, these vectors would also be altered in such a way as to render them non-pathogenic. In addition to the use of an appropriately mutated virus, implantation of transfected multipotent neural cell lines may also provide a means for delivery of the $\gamma_134.5$ gene to the CNS which avoids the blood brain barrier.

In addition, use of the HSV-1 virus with a specific mutation in the $\gamma_134.5$ gene provides a method of therapeutic treatment of tumorogenic diseases both in the CNS and in all other parts of the body. The " $\gamma_134.5$ minus" virus can induce apoptosis and thereby cause the death of the host cell, but this virus cannot replicate and spread. Therefore, given the ability to target tumors within the CNS, the $\gamma_134.5$ minus virus has proven a powerful

therapeutic agent for hitherto virtually untreatable forms of CNS cancer. Furthermore, use of substances, other than a virus, which inhibit or block expression of genes with anti-apoptotic effects in target tumor cells can also serve
5 as a significant development in tumor therapy and in the treatment of herpes virus infection, as well as treatment of infection by other viruses whose neurovirulence is dependent upon an interference with the host cells' programmed cell death mechanisms.

10

SUMMARY OF THE INVENTION

This invention relates to methods for the prevention or treatment of programmed cell death, or apoptosis, in neuronal cells for therapy in connection with neurodegenerative diseases, as well as methods of treatment
15 of cancer and other tumorogenic diseases and herpes virus infection. The present invention also relates to assay methodologies allowing for the identification of substances capable of modulating the effects of the γ ,34.5 gene or its protein expression product ICP34.5, i.e., substances
20 capable of potentiating or inhibiting their effects. Additionally, the present invention also relates to assay methodologies designed to identify candidate substances able to mimic either γ ,34.5 expression or the activity of ICP34.5. The present invention also relates to methods of
25 delivering genes to cells for gene therapy.

In one illustrative embodiment of the present invention a method of preventing or treating programmed cell death in neuronal cells is described in which a non-pathogenic vector is prepared which contains the γ ,34.5 gene. This vector is then introduced into neuronal cells
30 which are presently undergoing or are likely to undergo programmed cell death. Those skilled in the art will realize that several vectors are suitable for use in this method, although the present invention envisions the use of

certain unique and novel vectors designed specifically for use in connection with delivery of the γ ,34.5 gene.

One such vector envisioned by the present invention is the HSV-1 virus itself, modified so as to render it non-pathogenic. Because of the unique capability of the HSV-1 virus to use an axon's internal transport system to move from the peripheral nerve endings of the neuron into the neuronal cell body, the present invention proposes the use of the non-pathogenic HSV-1 virus injected into the vicinity of the synaptic terminals of affected neurons, or in the area of a peripheral wound or lesion or other appropriate peripheral locus. The HSV-1 virus containing the γ ,34.5 gene, under a different target-specific promoter, would then be transported into the neuronal cell body via retrograde axonal transport.

The present invention envisions specific genomic modifications being introduced into the HSV-1 virus in order to render the virus non-cytotoxic. These modifications could include deletions from the genome, rearrangements of specific genomic sequences, or other specific mutations. One example of such a modification comprises modification or deletion of the α 4 gene which encodes the ICP4 protein. Deletion or modification of the gene expressing ICP4 renders the HSV-1 virus unable to express genes required for viral DNA and structural protein synthesis. However, the γ ,34.5 gene placed under a suitable promoter would be expressed, thus inducing an anti-apoptotic effect in the neuron without the potential for stress induced neurovirulence. Other genes which might be modified include the the α 0 gene. The present invention also envisions the use of other vectors including, for example, retrovirus, picorna virus, vaccinia virus, HSV-2, coronavirus, eunyavirus, togavirus or rhabdovirus vectors. Again, use of such viruses as vectors will necessitate

construction of deletion mutations so that the vectors will be safe and non-pathogenic.

Another method by which the present invention envisions introducing the γ ,34.5 gene into neuronal cells undergoing or likely to undergo programmed cell death, is through the use of multi-potent neural cell lines. Such lines have been shown to change phenotype in vitro and have also been demonstrated to become integrated into the central nervous system of mice and to differentiate into neurons or glia in a manner appropriate to their site of engraftment. Snyder, et al., Cell, 68: 33-51, 1992. Transplant or engraftment of multi-potent neural cell lines into which the γ ,34.5 gene has been introduced into an area of the central nervous system in which cells are undergoing or are likely to undergo programmed cell death is expected to lead to reversal and inhibition of programmed cell death.

It is expected that the ability of γ ,34.5 to inhibit apoptosis will be a boon not only in human medicine, but also in basic scientific research. In this regard the present invention also envisions the use of the γ ,34.5 gene in the extension of the life of neuronal cells in cell culture. Introduction of a non-cytotoxic vector into cultured neuronal cells will have an anti-apoptotic effect and will thereby extend the life of cell cultures. This in turn will extend the time periods over which experimentation may be conducted, and can also be expected to decrease the cost of conducting basic research.

In addition to utilizing a vector comprising the γ ,34.5 gene, the present invention also discloses a method of preventing or treating programmed cell death in neuronal cells which involves the use of the product of expression of the γ ,34.5 gene. The protein expressed by γ ,34.5 is called ICP34.5. Ackermann, et al. (J. Virol., 58: 843-850,

1986) reported that ICP34.5 has an apparent molecular weight of 43,500 upon SDS-polyacrylamide gel electrophoresis, appears to accumulate largely in the cytoplasm of HIV infected cells, and in contrast to many 5 HSV-1 proteins, ICP34.5 has been demonstrated to be soluble in physiologic solutions.

In practicing this method or the method in which the γ ,34.5 gene is introduced into cells, it is envisioned that the γ ,34.5 gene or a biological functional equivalent thereof could be used for gene therapy, or ICP34.5 in a purified form or a biological functional equivalent of the ICP34.5 protein could be utilized as an anti-apoptotic agent. As used herein, functional equivalents are intended to refer to those proteins, and their encoding 10 nucleic acid sequences, in which certain structural changes have been made but which nonetheless are, or encode, 15 proteins evidencing an effect similar to that of ICP34.5.

In light of the fact that certain amino acids may be substituted for other amino acids in a protein without appreciable loss of defined functional activity, it is contemplated by the inventors that various changes may be made in the sequence of the ICP34.5 protein (or the underlying DNA of the γ ,34.5 gene) without an appreciable loss of biological utility or activity. Amino acids with 20 similar hydropathic scores may be substituted for one another (see Kyte et al., J. Mol. Biol., 157: 105-132, 1982, incorporated herein by reference), as may amino acids with similar hydrophilicity values, as described in U.S. 25 Patent 4,554,101, incorporated herein by reference.

Therefore, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary 30 substitutions which take various of the foregoing

characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

5 This embodiment of the present invention describes a method which involves combining ICP34.5 or a biological functional equivalent thereof with a pharmaceutically acceptable carrier in order to form a pharmaceutical composition. (It should be understood in subsequent
10 discussions that when γ 34.5 or ICP34.5 are referred to, the inventors intend to include biological functional equivalents, including any chemicals which mimic the effect of γ 34.5.) Such a composition would then be administered to neurons likely to undergo or undergoing programmed cell
15 death. Such a composition could be administered to an animal using intravenous, intraspinal injection or, in certain circumstances, oral, intracerebral or intraventricular administration may be appropriate. Furthermore, neuronal cells in culture could also benefit
20 from administration of ICP34.5 through administration directly into the medium in which the neuronal cells are grown.

ICP34.5 can be prepared using a nucleic acid segment which is capable of encoding ICP34.5 (i.e., the γ 34.5 gene
25 or a biological functional equivalent). Such a segment could be expressed using, for example, a technique involving transferring the γ 34.5 segment into a host cell, culturing the host cell under conditions suitable for expression of the segment, allowing expression to occur,
30 and thereafter isolating and purifying the protein using well established protein purification techniques. The nucleic acid segment would be transferred into host cells by transfection or by transformation of a recombinant vector into the host cell.

A particularly important embodiment of the present invention relates to assays for candidate substances which can either mimic the effects of the γ ,34.5 gene, or mimic the effects of ICP34.5, as well as assays for candidate substances able to potentiate the function of γ ,34.5 or potentiate the protective function of ICP34.5. Additionally, methods for assaying for candidate substances able to inhibit either γ ,34.5 expression or the activity of ICP34.5 are also embodiments of the present invention.

In an exemplary embodiment, an assay testing for candidate substances which would block the expression of the anti-apoptosis gene or inhibit the activity of an anti-apoptotic protein such as ICP34.5 would proceed along the following lines. A test plasmid construct bearing the α sequence promoter and portions of the coding sequence of γ ,34.5 is fused to the lacZ reporter gene, or any other readily assayable reporter gene. This construct is then introduced into an appropriate cell line, for example a neuroblastoma or PC12 cell line, by G418 selection. A clonal and continuous cell line for screening purposes is then established. A control plasma construct bearing an HSV late promoter (a promoter which would normally not be expressed in cell lines and not induced to express by a stress factor which would normally induce apoptosis) is fused to the same or different indicator gene. This construct is also introduced into a continuous clonal cell line and serves as a control for the test cell line. The anti-apoptosis drugs would then be applied. Environmental stresses which typically trigger α sequence promoter activation and cause programmed cell death, such as UV injury, viral infection or deprivation of nerve growth factor, would then be applied to the cells. In control cells, the stress should have no effect on the cells and produce no detectible reaction in the assay. Stress in a test cell line in the absence of a positive candidate substance would give rise to an appropriate reaction,

typically a colorimetric reaction. Introduction of stress to the test cell line in the presence of the candidate substance would give rise to an opposite colorimetric reaction indicating that the candidate substance 5 interferes either with expression of the γ ,34.5 gene, or with the ability of the substance to interfere with the anti-apoptotic activity of ICP34.5.

Similarly, the present invention describes an assay for candidate substances which would mimic or potentiate 10 the activity of ICP34.5, or which would mimic the expression of γ ,34.5, and such an assay would proceed along lines similar to those described above. A test cell line (e.g., a neuroblastoma cell line) constitutively expressing ICP34.5 and a fluorescent tagged cellular gene or any other 15 tag providing an easily detected marker signalling viability of the cells is produced. In addition, a corresponding null cell line consisting of an appropriate indicator gene, for example the α -lacZ indicator gene, and the same host indicator gene as in the test cell line is 20 also produced. Also, a third cell line (e.g., a vero cell line) consisting of the same indicator gene and the identical host indicator gene is also produced. Again, environmental stresses which trigger programmed cell death in the absence of γ ,34.5 are applied to the cells. 25 Candidate substances are also applied in order to determine whether they are able to mimic or potentiate the anti-apoptotic effects of γ ,34.5 expression or the anti-apoptotic activity of ICP34.5 or biological functional equivalents thereof.

30 The present invention also embodies a method of delivering genes for gene therapy. In an exemplary embodiment, the method involves combining the gene used for gene therapy with a mutated virus such as those described above, or with the HSV-1 virus rendered non-pathogenic. 35 The gene and the virus are then combined with a

pharmacologically acceptable carrier in order to form a pharmaceutical composition. This pharmaceutical composition is then administered in such a way that the mutated virus containing the gene for therapy, or the HSV-1 5 wild type virus containing the gene, can be incorporated into cells at an appropriate area. For example, when using the HSV-1 virus, the composition could be administered in an area where synaptic terminals are located so that the virus can be taken up into the terminals and transported in 10 a retrograde manner up the axon into the axonal cell bodies via retrograde axonal transport. Clearly, such a method would only be appropriate when cells in the peripheral or central nervous system were the target of the gene therapy.

The present invention also envisions methods and 15 compositions for the treatment of cancer and other tumorogenic diseases, as well as herpes infections or other infections involving viruses whose virulence is dependent upon an anti-apoptotic effect. Candidate substances identified as having an inhibiting effect upon either the 20 expression or activity of ICP34.5 identified in the assay methods discussed above could be used to induce cell death in target tumor cells, or in virus-infected cells. Pharmaceutical compositions containing such substances can be introduced using intrathecal, intravenous, or direct 25 injection into the tumor or the infected area, as appropriate.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows DNA sequence comparisons of HSV-1 strains F (SEQ ID NO's 1-5), 17syn+ (SEQ ID NO's: 6-9), MGH-10 (SEQ 30 ID NO's 10-15), and CVG-2 (SEQ ID NO's 16-20) in the region of the gene for ICP34.5 (left panel) and the predicted open frames for ICP34.5 in these strains (SEQ ID NO's 25-34) (right panel). Unless otherwise indicated by a new base (insertion of A, C, G, or T), a new amino acid (three-letter code), or absence of a base or amino acid (-), the 35

Sequences for strains HSV-1(17)syn+, HSV-1(MGH-10), and HSV-1(CVG-2) were identical to the sequence for HSV-1(F). An asterisk indicates initiation of a repeat sequence of nine nucleotides or three amino acids. Direct repeat 5 1(DR1) designates the 20-base-pair repeat sequence flanking the a sequence. Sequences upstream of direct repeat 1 are contained within the a sequence. The number at the end of each line indicates the relative position from nucleotide 1 (left panel) or amino acid 1 (right panel). The initiation 10 and termination codons for the HSV-1(F) sequence are underlined.

Fig. 2 shows sequence arrangements of the genome of wild-type strain HSV-1 strain F [HSV-1(F)] and of recombinant viruses derived from it. Top line, the 15 sequence arrangement of HSV-1(F) Δ305. The rectangles identify the inverted repeats ab, b' a' c, and ca. The HSV-(F) a sequence is present in a direct orientation at the two genomic termini and in the inverted orientation at the junction between the long and short components. The b 20 and c sequences are approximately 9 and 6 kbp long, respectively. The triangle marked TK identifies the position of the tk gene and of the Bgl II to Sac I sequence of BamHI Q fragment deleted from HSV-1(F)Δ305. Lines two and three from the top show that the b sequences contain 25 the genes specifying ICP34.5 and ICP0 and, since b sequence is repeated in an inverted orientation, there are two copies of these genes per genome. The construction of the a24-tk fragment containing portions of the glycoprotein H gene has been described. Chou and Roizman, J. Virol., 57: 30 (1986); Ackerman et al., J. Virol., 58: 843 (1986); Chou and Roizman, J. Virol., 64: 1014 (1990). Line 7 shows a schematic diagram of the insertion of the oligonucleotide containing stop codons in all three reading frames. The plasmids pRB3615 and pRB2976 used in the construction of 35 R4002 and R4004, respectively, were described elsewhere. Chou and Roizman, J. Virol., 57: 629 (1986) and J. Virol.,

64: 1014 (1990). To generate pRB3616, plasmid pRB143 was digested with BstEII and Stu I, blunt-ended with T4 polymerase, and religated. The asterisks designate nucleotides from vector plasmid that form cohesive ends with the synthesized oligomers (SEQ ID NO's 21-22). The insertion of the α 4 epitope into the first amino acid of ICP34.5 (line 9) has been described, Chou and Roizman, J. Virol., 64: 1014 (1990), except that in this instance the sequence was inserted into both copies of the γ 34.5 gene (SEQ ID NO's 23, 24 and 34). The tk gene was restored in all recombinant viruses tested in mice. HSV-1(F)R (line 6) was derived from R3617 by restoration of the sequences deleted in γ 34.5 and tk genes. N, Be, S, and St are abbreviations for Nco I, BstEII, Sac I, and Stu I restriction endonucleases (New England Biolabs), respectively. The numbers in parentheses are the tk⁺ version of each construct tested in mice.

Fig. 3 shows an autoradiographic image of electrophoretically separated digest of plasmid, wild-type, and mutant virus DNAs, transferred to a solid substrate and hybridized with labelled probes for the presence of γ 34.5 and tk genes. The plasmids or viral DNAs shown were digested with BamHI or, in the case of R4009 shown in lanes 10, with both BamHI and Spe I. The hybridization probes were the fragment Nco I to Sph I contained entirely within the coding sequences of γ 34.5 (left panel) and the BamHI Q fragment of HSV-1(F) (right panel). The probes were labeled by nick translation of the entire plasmid DNAs with [α -³²P] deoxycytidine triphosphate and reagents provided in a kit (Du Pont Biotechnology Systems). The DNAs that were limit digested with BamHI (all lanes) or both BamHI and Spe I (left panel, lane 10) were electrophoretically separated on 0.8% agarose gels in 90 mM trisphosphate buffer at 40 V overnight. The DNA was then transferred by gravity to two nitrocellulose sheets sandwiching the gel and hybridized overnight with the respective probes. γ 34.5 maps in BamHI

S and SP fragments, which form a characteristic ladder of bands at 500-bp increments. The ladders are a consequence of a variable number of α sequences in the repeats flanking the unique sequences of the junction between the long and 5 short components, whereas BamHI S is the terminal fragment of the viral genome at the terminus of the long component, whereas BamHI SP is a fragment formed by the fusion of the terminal BamHI S fragment with BamHI P, the terminal BamHI fragment of the short component. Bands of BamHI S, SP, and 10 Q and their deleted versions, Δ BamHI S, Δ BamHI SP, and Δ BamHI Q (Δ Q), respectively, are indicated. Band 1 represents the 1.7-kbp α 27-t_A insert into the BamHI SP fragment in R4002, and therefor this fragment reacted with both labeled probes (lanes 4). Band 2 represents the same 15 insertion into the BamHI S fragment.

Fig. 4 shows autoradiographic images (left panel) and photograph of lysates of cells mock infected (M) or infected with HSV-1(F) and recombinant viruses (right panel) separated electrophoretically in denaturing 20 polyacrylamide (10%) gels, transferred electrically to a nitrocellulose sheet, and stained with rabbit polyclonal antibody R4 described elsewhere. Ackerman et al., J. Virol., **58**: 843 (1986); Chou and Roizman, J. Virol., **64**: 1014 (1990). Replicate cultures of Vero cells were 25 infected and labeled with [³⁵S]methionine (Du Pont Biotechnology Systems) from 12 to 24 hours after infection, and equivalent amounts of cell lysates were loaded in each slot. The procedures were as described (Ackerman et al.; Chou and Roizman) except that the bound antibody was made 30 apparent with the alkaline phosphatase substrate system supplied by Promega, Inc. Infected cell proteins were designated by number according to Honess and Roizman (J. Virol., **12**: 1346 (1973)). The chimeric ICP34.5 specified by R4003 migrated more slowly than the protein produced by 35 other viruses because of the increased molecular weight caused by the insertion of the epitope.

Fig. 5 is a schematic representation of the genome structure and sequence arrangements of the HSV-1 strain F [HSV01(F)] and related mutants. Top line: The two covalently linked components of HSV-1 DNA, L and S, each consist of unique sequences flanked by inverted repeats (7, 31). The reiterated sequences flanking the L component designated as ab ad b'a' are each 9 kb in size, whereas the repeats flanking the S component are 6.3 kb in size (31). Line 2: expansion of portions of the inverted repeat sequences ab and b'a' containing the $\gamma_134.5$ and $\alpha 0$ genes. Line 3: sequence arrangement and restriction endonuclease sites in the expanded portions shown in line 2. Open box represents the 20 bp direct repeat sequence (DR1), flanking the a sequence (26,27). Restriction site designations are N,- NcoI; Be,- BstEII; S,- SacI; St,- StuI. Line 4: the thin line and filled rectangle represent the transcribed and coding domains of the $\gamma_134.5$ gene (406). Vertical line, location of the transcription initiation sites of $\gamma_134.5$ and of $\alpha 0$ genes. In the R3616 viral recombinant, one Kb was deleted between BstEII at 28th amino acid of $\gamma_134.5$ to StuI at the 3' terminus of the genes as shown. In HSV-1(F)R DNA, the sequences deleted from the $\gamma_134.5$ gene in R3616 were restored and therefore the virus could be expected to exhibit a wild-type phenotype. The R4009 recombinant virus DNA contains an in frame translation termination codons at the BstEII site. Vertical arrow on top points to the site of the stop codon insertion.

Fig. 6 shows an autoradiographic image of electrophoretically separated lysates of infected cells labeled for 90 minutes with ^{35}S -methionine at stated time points. The SK-N-SH neuroblastoma and Vero cell lines were mock infected (M) or exposed at 37°C to 5 pfu of wild-type or mutant viruses in 6 well (Costar, Cambridge, Mass.) dishes. At 2 hours post exposure, the cells were overlaid with mixture 199 supplemented with 1% calf serum. At 5.5 and 11.5 hours post exposure of cells to viruses, replicate

infected 6 well cultures were overlaid with 1 ml of the 199v medium lacking unlabeled methionine but supplemented with 50 μ Ci of 35 S-methionine (specific activity >1,000 Ci/mmol, Amersham Co. Downers Grove, IL). After 90 minutes in labeling medium, the cells were harvested, solubilized in a buffer containing sodium dodecyl sulphate, subjected to electrophoresis on a denaturing 12% polyacrylamide gels crosslinked with N, N' Diallyltartardiamide, electrically transferred to nitrocellulose sheet and subjected to autoradiography as previously described (13). Infected cell polypeptides (ICP) were designated according to Honess and Roizman, J. Virol., 12: 1347-1365 (1973).

Fig. 7 shows autoradiographic images of labeled polypeptides electrophoretically separated in denaturing gels and photographs of protein bands made apparent by their reactivity with antibodies. The SK-N-SH neuroblastoma and Vero cells were either mock infected (M) or infected with 5 pfu of either R3616 or the parent HSV-1(F) per cell as described in the legend to Figure 6. The cultures were labeled for 1.5 hr before harvesting at 13th hr post exposure of cells to virus. Preparation of cell extracts, electrophoresis of the polypeptides, electric transfer of the separated polypeptides to a nitrocellulose sheet, and autoradiography were carried out as described elsewhere (Ackerman et al., J. Virol., 52: 108-118, 1984). The nitrocellulose sheets were reacted with the respective antibodies with the aid of kits from Promega, Inc. (Madison, WI) according to manufacturer's instruction. Monoclonal antibodies H1142 against α 27 and H725 against the product of the U_L26.5 gene were the generous gift of Lenore Pereira, University of California at San Francisco. The M28 monoclonal antibody to U_S11 protein and the rabbit polyclonal antibody R161 against viral thymidine kinase (β tk) were made to a specific peptide (M. Sarmiento and B.

Roizman, unpublished studies) in this laboratory. ICP designations were the same as noted before.

Fig. 8 shows an autoradiographic image of viral proteins expressed during infection on SK-N-SH neuroblastoma cell lines in the presence or absence of phosphonoacetate (PAA). Duplicate SK-N-SH neuroblastoma cell cultures were either treated with phosphonoacetate (300 µg/ml; Sigma Chemical Co., St. Louis, MO) starting at 1.5 hr prior to infection continuously until the termination of infection or left untreated. The cultures were either mock infected or exposed to 5 pfu of either HSV-1(F), R3616, R4009 and HSV-1(F)R at 5 pfu/cell. At 11.5 hours post exposure to virus, the cells were overlaid with medium containing 50 µCi of ³⁵S-methionine as described in legend to Figure 6. Polypeptide extraction, electrophoresis on 12% polyacrylamide gels crosslinked with N,N' Diallyltartardiamide, electrical transfer to nitrocellulose sheets and autoradiography were as described in the legend to Figure 6.

Fig. 9 shows viral DNA and RNA accumulation in infected SK-N-SH neuroblastoma and Vero cell cultures. Left panel: Photograph of ethidium bromide stained agarose gel containing electrophoretically separated BamHI digests of total DNAs extracted from mock-infected cells or cells infected with HSV-1(F), R3616, R4009 or HSV-1(F)R viruses. Right panel: Hybridization of electrophoretically separated RNA transferred to a nitrocellulose sheets probed with RNA sequences antisense to α 47, U_s10 and U_s11 open reading frames. SK-N-SH neuroblastoma and Vero cells were either mock-infected or exposed to 5 pfu of HSV-1(F) or of R3616 per cell. Total DNAs were extracted from cells at 17 hr post infection by the procedure published by Katz et al., (J. Virol., 64: 4288-4295), digested with BamHI, electrophoresed in 0.8% agarose gel at 40V overnight and stained with ethidium bromide for visualization. For RNA

analysis, SK-N-SH neuroblastoma and Vero cells were either mock-infected or infected with R3616 and HSV-1(F) as described above. At 13 hrs post exposure of cells to virus the RNA was extracted by the procedure of Peppel and Baglioni (BioTechniques, 9: 711-712, 1990). The RNAs were then separated by electrophoresis on 1.2% agarose gel, transferred by gravity to a nitrocellulose sheet and probed with anti-sense RNA made from in vitro transcription of pRB3910 off T7 promoter using kit from Promega, Inc. according to manufacturer's instruction. α 47, U_S10 and U_S11 transcripts overlap in sequence and share the same 3' co-terminal sequence. McGeoch et al., J. Gen. Virol., 64: 1531-1574 (1988). The U_S10 transcript is of low abundance and not detected in this assay.

15 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
Introduction

The present invention relates to the use of the HSV-1 γ 134.5 gene, the ICP34.5 protein expressed by that gene, and derivatives of the protein which function in a similar manner as therapeutics for (neuronal) programmed cell death. The present invention also relates to the use of altered, non-pathogenic HSV-1 virus (as well as other viruses) as a vector for gene therapy. Other aspects of the present invention relate to assays for detecting candidate substances capable of acting as anti-apoptotic agents, as well as assays for detecting candidate substances able to induce programmed cell death in tumor cells. Additionally, the present invention also relates to methods for treating cancer and other tumorigenic diseases. Finally, the present invention also relates to the use of candidate substances capable of inactivating the γ 134.5 gene or ICP34.5 and thereby suppressing HSV-1 and other viral infections.

35 The wild-type HSV-1 genome (150 kilobase pairs) has two components, L and S, each possessing unique sequences

flanked by inverted repeats. The repeat sequences of the L component, designated *ab* and *b'a'*, are each 9 kilobase pairs, whereas the repeat sequences of the S component, designated *a'c'* and *ca*, are each 6.5 kilobase pairs.

5 Wadsworth et al., J. Virol., 15: 1487-1497 (1975). The shared *a* sequence, 500-base pairs long in HSV-1 strain F [HSV-1(F)], is present in one copy at the S component terminus and in one to several copies, in the same orientation, at the junction between L and S components.
10 The L and S components invert relative to each other such that the DNA extracted from virions or infected cells consists of four isomers differing solely in the orientation of the L and S components relative to each other. Hayward et al., Proc. Natl. Acad. Sci. USA, 72:
15 4243-4247 (1975). The *a* sequence appears to be a *cis*-acting site for inversions inasmuch as insertion of the *a* sequence elsewhere in the genome or deletion of the entire internal inverted repeat sequences (*b'a'c'*) leads to additional inversions or the loss of the ability of the L
20 and S components to invert, respectively. The *a* sequence was also shown to contain the *cis*-acting sites for the circularization of the genome after infection, for cleavage of the HSV genome from concatemers, and for encapsidation of the DNA.

25 HSV-1 genomes contain at least 73 genes whose expression is coordinately regulated and sequentially ordered in cascade fashion. The α genes are expressed first, followed by β , γ_1 and γ_2 genes. The differentiation among β , γ_1 and γ_2 genes is operationally based on the
30 effect of inhibitors of viral DNA synthesis. Whereas the expression of β genes is stimulated and that of γ_1 genes is only slightly reduced by inhibitors of viral DNA synthesis, the expression of γ_2 genes stringently requires viral DNA synthesis.

In the course of studies on the function of the a sequence, Chou and Roizman (Cell, 41: 803-811, 1985) noted that the chimeric structure consisting of the a sequence fused to the 5' transcribed, noncoding sequences of the thymidine kinase (TK) gene of HSV-1 was inducible in transferred cells and regulated as a γ_1 gene when inserted into the viral genome. This observation suggested that the terminus of the a sequence nearest the b sequence of the inverted repeats contained a promoter and the transcription initiation site of a gene whose structural sequences were located in the b sequences flanking the L component. Studies involving hybridization of labeled DNA probes to electrophoretically separated RNAs extracted from infected cells, and S1 nuclease analyses confirmed the existence of RNA transcripts initiating in the a sequence. Nucleotide sequence analyses revealed the presence of an open reading frame capable of encoding a protein 263 amino acids long. Chou and Roizman, J. Virol., 64: 1014-1020 (1990).

Previous studies have shown that each inverted repeat of the S component contains in its entirety a gene designated $\alpha 4$, whereas each of those of the L component contains in its entirety a gene designated $\alpha 0$. See, e.g., Mackem and Roizman, J. Virol., 44: 934-947 (1982). The putative gene identified on the basis of nucleotide sequence and analyses of RNA is also present in two copies per genome. Because of the overlap of the domain of this gene with the a sequence containing the *cis*-acting sites for inversion, cleavage of DNA from concatemers, and packaging of the DNA, it was of interest to identify and characterize the gene product. For this purpose, the observation that the nucleotide sequence predicted the presence in the protein of the amino acid triplet Ala-Thr-Pro repeated 10 times was utilized, and antibody to a synthetic peptide synthesized on the basis of this sequence reacted with a 43,500-apparent-molecular-weight HSV-1 protein. Ackerman et al., J. Virol., 58: 843-850, 1986.

The extent of variability of the open reading frame that encodes ICP34.5 was established by comparing the nucleotide sequences of three HSV-1 strains passaged a limited number of times outside a human host. Chou and 5 Roizman (J. Virol., 64: 1014-1020, 1990) reported that the gene that specifies ICP34.5 contains 263 codons conserved in all three limited passage strains but not in the reported sequence of the HSV-1(17)syn+ strain. (FIG. 1) To ensure that the antibody to a predicted repeat sequence, 10 Ala-Thr-Pro, reacted with ICP34.5 rather than with a heterologous protein with a similar repeat sequence, a short sequence of 45 nucleotides that encodes an epitope characteristic of another HSV-1 gene was inserted near the 5' terminus of the ICP34.5-coding domain. The recombinant 15 virus expressed a protein with an appropriately slower electrophoretic mobility and which reacted with both the monoclonal antibody to the inserted epitope and rabbit antiserum to the Ala-Thr-Pro repeat element.

Studies of the identification of the genes associated 20 with neurovirulence have repeatedly implicated DNA sequences located at or near a terminus of the long component of HSV-1 DNA. Thus, Centifanto-Fitzgerald et al. (J. Exp. Med., 155: 475, 1982) transferred, by means of a DNA fragment, a virulence marker from a virulent to an 25 antivirulent strain of HSV-1. Deletion of genes located at one terminus of the long component of HSV-1 DNA contributed to the lack of virulence exhibited by a prototype HSV vaccine strain. Meignier et al., J. Infect. Dis., 158: 602 (1988). In other studies, Javier et al. (J. Virol., 65: 30 1978, 1987) and Thompson et al. (Virology, 172: 435, 1989) demonstrated that an HSV-1 x HSV-2 recombinant virus consisting largely of HSV-1 DNA but with HSV-2 sequences located at one terminus of the long component was 35 avirulent; virulence could be restored by rescue with the homologous HSV-1 fragment. Taha et al. (J. Gen. Virol., 70: 705, 1989) described a spontaneous deletion mutuant

lacking 1.5 kbp at both ends of the long component of a HSV-2 strain. Because of heterogeneity in the parent virus population, the loss of virulence could not be unambiguously related to the specific deletion, although 5 the recombinant obtained by marker rescue was more virulent than the deletion mutant. In neither study was a specific gene or gene product identified at the mutated locus, and no gene was specifically linked to virulence phenotype.

Role of the $\gamma_{134.5}$ Gene

10 To test the possible role of the product of the $\gamma_{134.5}$ gene, ICP34.5, a series of four viruses (Fig. 2) were genetically engineered by the procedures of Post and Roizman (*Cell*, 25: 227, 1981, incorporated herein by reference).

15 1) Recombinant virus R4002 (Fig. 4, lane 3) contained the insertion of a thymidine kinase (*tk*) gene driven by the promoter of the $\alpha27$ gene ($\alpha27-tk$) in both copies of the ICP34.5 coding sequences. It was constructed by cotransfected rabbit skin cells with intact DNA of HSV-
20 1(F) Δ 305, a virus from which a portion of the *tk* gene was specifically deleted, with the DNA of plasmid pRB3615, which contains the $\alpha27-tk$ gene inserted into the $\gamma_{134.5}$ gene contained in the BamHI S fragment. Recombinants that were *tk*⁺ were then selected on human 143 thymidine kinase minus
25 (TK⁻) cells. The fragment containing the $\alpha27-tk$ gene contains downstream from the *tk* gene: the 5' untranscribed promoter, the transcribed noncoding sequence, and the initiating methionine codon of the glycoprotein H gene. The BstEII site into which the $\alpha27-tk$ fragment was inserted
30 is immediately upstream of the codon 29 of the $\gamma_{134.5}$ open reading frame. As a consequence, the initiating codon of glycoprotein H was fused in frame and became the initiating codon of the truncated open reading frame of the $\gamma_{134.5}$ gene

(Fig. 2, line 3). The recombinant selected for further study, R4002, was shown to contain the α 27-tk gene insert in both copies of γ ,34.5 gene (Fig. 3, lanes 4) and specified only the predicted truncated product of the chimeric γ ,34.5 gene (Fig. 4, right panel, lane 3). The amounts of the native ICP34.5 protein detected in these and previous studies have been generally low. The chimeric genes formed by the fusion of the 5' transcribed noncoding region and the initiating codon of glycoprotein H in frame with the truncated γ ,34.5 gene were expressed far more efficiently than the native genes.

2) The recombinant virus R3617 (Fig. 2, line 5 from the top) lacking 1 kb of DNA in each copy of the γ ,34.5 gene was generated by cotransfected rabbit skin cells with intact R4002 DNA and the DNA of plasmid pRB3616. In this plasmid, the sequences containing most of the coding domain of γ ,34.5 has been deleted (Fig. 2, line 5 from top). The tk⁻ progeny of the transfection was plated on 143TK⁻ cells overlaid with medium containing bromodeoxy uridine (BrdU). This procedure selects tk⁻ viruses, and since the tk gene is present in both copies of the γ ,34.5 gene, the selected progeny of the transfection could be expected to contain deletions in both copies. The selected tk⁻ virus designated as R3617 was analyzed for the presence of the deletion in both copies of the γ ,34.5 gene. For assays of neurovirulence, the deletion in the native tk⁻ gene of R3617, which traces its origin from HSV-1(F) Δ 305, had to be repaired. This was done by cotransfection of rabbit skin cells with intact R3617 DNA and BamHI Q fragment containing the tk gene. The virus selected for tk⁺ phenotype in 143TK⁻ cells was designated R3616. This virus contains a wild-type BamHI Q fragment (Fig. 3, right panel, lane 6) and does not make ICP34.5 (Fig. 4, right panel).

3) To ascertain that the phenotype of R3616 indeed reflects the deletion in the γ ,34.5 gene, the deleted

sequences were restored by cotransfected rabbit skin cells with intact R3617 DNA, the HSV-1(F) BamHI Q DNA fragment containing the intact tk gene, and the BamHI SP DNA fragment containing the intact γ 134.5 gene in the molar ratios of 1:1:10, respectively. Viruses that were tk⁺ were then selected in 143TK⁻ cells overlaid with medium containing hypoxanthine, aminopterin, and thymidine. The tk⁺ candidates were then screened for the presence of wild-type tk and γ 134.5 genes. As expected, the selected virus designated HSV-1(F)R (Fig. 2, line 6) contained a wild-type terminal long component fragment (compare Fig. 3, left panel, lanes 2, 7, and 8), and expressed ICP34.5 (Fig. 4, right panel, lane 6).

4) To eliminate the possibility that the phenotype of R3616 reflects deletion in cryptic open reading frames, a virus was constructed (R4010, Fig. 2, line 7 from the top) that contains translational stop codons in all three reading frames in the beginning of the ICP34.5 coding sequence. The 20-base oligonucleotide containing the translational stop codons and its complement sequence (Fig. 2) were made in an Applied Biosystems 380D DNA synthesizer, mixed at equal molar ratio, heated to 80°C, and allowed to cool slowly to room temperature. The annealed DNA was inserted into the HSV-1(F) BamHI S fragment at the BstEII site. The resulting plasmid pRB4009 contained a stop codon inserted in the beginning of the ICP34.5 coding sequence. The 20 nucleotide oligomer DNA insertion also contained a Spe I restriction site, which allowed rapid verification of the presence of the insert. To generate the recombinant virus R4010, rabbit skin cells were cotransfected with the intact DNA of R4002 and the pRB4009 plasmid DNA. Recombinants that were tk⁻ were selected in 143TK⁻ cells in medium containing BrdU. The tk⁺ version of this virus, designated R4009, was generated by cotransfection of intact tk⁻ R4010 DNA with HSV-1(F) BamHI Q DNA fragment, and selection of tk⁺ progeny. The virus selected for

neurovirulence studies, R4009, contained the Spe I restriction endonuclease cleavage site in both BamHI S and SP fragments (compare Fig. 3, left panel, lanes 9 and 10) and did not express ICP34.5 (Fig. 4, right panel, lane 7).

5 5) R4004 (Fig. 2, last line) was a recombinant virus produced by insertion of a sequence encoding 16 amino acids. This sequence has been shown to be the epitope of the monoclonal antibody H943 reactive with a viral protein designated as ICP4. Hubenthal-Voss et al., J. Virol., 62: 10 454 (1988). The virus was generated by cotransfected intact R4002 DNA and the DNA of plasmid pRB3976 containing the insert, and the selected tk⁻ progeny was analyzed for the presence of the insert. For neurovirulence studies, its tk gene was restored (recombinant virus R4003) as 15 described above. The DNA sequence was inserted in frame at the Nco I site at the initiating methionine codon of the γ_1 34.5 gene. The insert regenerated the initiating methionine codon and generated a methionine codon between the epitope and the remainder of ICP34.5. Because of the 20 additional amino acids, the protein migrated more slowly in denaturing polyacrylamide gels (Fig. 4, right panel, lane 4).

Plaque morphology and size of all of the recombinants were similar to those of the wild-type parent, HSV-1(F) 25 when plated on Vero, 143TK⁻, and rabbit skin cells lines. Whereas HSV-1(F)R and R4003 replicated as well as the wild-type virus in replicate cultures of Vero cells, the yields of R3616 and R4009 were reduced to one-third to one-fourth the amount of the wild type. Although ICP34.5 was not 30 essential for growth of HSV-1 in cells in culture, the results of the studies shown in Table 1 indicate that the deletion or termination of translation of the γ_1 34.5 has a profound effect on the virulence of the virus. Thus, all 35 of the mice inoculated with the highest concentration [1.2 $\times 10^6$

plaque-forming units (PFU)] of R3616 survived. In the case of R4009, only three of ten mice died as a result of inoculation with the highest concentration of virus ($\sim 10^7$ PFU). In comparison with other deletion mutants, R3616 and 5 R4009 rank among the least pathogenic viruses reported to date. The virus in which the $\gamma_134.5$ gene was restored exhibited the virulence of the parent virus.

TABLE 1

	Virus in the inoculum	Genotype	PFU/LD ₅₀
10	HSV-1(F)	Wild-type parent virus	420
	R3616	1000-bp deletion in the $\gamma_134.5$	>1,200,000
	HSV-1(F)R	Restoration of $\gamma_134.5$ and tk	130
	R4009	Stop codon in $\gamma_134.5$	>10,000,000
	R4003	Monoclonal antibody epitope inserted at the NH ₂ terminal	4,200

15 20 Comparative ability of wild-type and recombinant viruses to cause death after intracerebral inoculation of mice. The neurovirulence studies were done on female BALB/C mice obtained at 21 days of age (weight \pm 1.8 g) from Charles River Breeding Laboratories in Raleigh, North Carolina. The viruses were diluted in minimum essential medium containing Earle's salts and 10% fetal bovine serum, penicillin, and gentamicin. The 25 mice were inoculated intracerebrally in the right cerebral hemisphere with a 26-gauge needle. The volume delivered was 0.03 ml, and each dilution of virus was tested in groups of ten mice. The animals were checked daily for mortality for 21 days. The LD₅₀ was calculated with the aid of the 30 "Dose effect Analysis" computer program from Elsevier Biosoft, Cambridge, United Kingdom.

35

The wild-type virus and all of the recombinants have identical surface glycoproteins necessary for attachment and penetration into brain cells. Injection of 10^6 PFU into the brain should result in infection and death of a significant number of the brain cells. Death after intracerebral inoculation results from viral replication, spread from cell to cell, and cell destruction before the immune system has a chance to act. Titrations of brain tissue suspended in minimal essential medium containing Eagle's salts and 10% fetal bovine serum showed that the brains of animals inoculated with the viruses that failed to make ICP34.5 contained very little virus. Thus, for the R3616 and R4009 viruses, the recovery was 120 and 100 PFU per gram of brain tissue, respectively. Given the amount of virus in the inoculum (highest concentration tested), it is not clear whether the small amounts of recovered virus represent a surviving fraction of the inoculum or newly replicated virus. In contrast, the amounts of virus recovered from mice inoculated with HSV-1(F)R and R4003 were 6×10^6 , respectively. These results indicate that the failure of the two recombinant viruses to cause death must be related to poor spread of virus in neuronal tissue as a consequence of the inability of mutant viruses to replicate in the CNS, reflecting a reduction in their host range.

In the course of studies designed to determine the function of the γ ,34.5 gene product, it was discovered that infection of cells of neuronal origin with mutants incapable of expressing the γ ,34.5 gene resulted in shutoff 5 of cellular protein synthesis, whereas infection of cells of non neuronal origin with wild type or mutant viruses resulted in sustained protein synthesis and production of infectious progeny.

10

**EXAMPLE 1 - IMPACT OF γ ,34.5
EXPRESSION ON PROGRAMMED CELL DEATH**

Materials and Methods

Cells Vero cells originally obtained from ATCC were propagated in DME media containing 5% calf serum. The human SK-N-SH neuroblastoma (NB) cell line was obtained 15 from ATCC (HTB11) and propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Viruses The isolation of herpes simplex virus 1 strain F, [HSV-1(F)] has been described by Ejercito et al. (J. Gen. Virol., 2: 357-364 (1968) (incorporated herein by 20 reference). The construction of recombinant viruses R3616, R4009, and HSV-1(F) was reported by Chou et al. (Science, 250: 1262-1266, November 30, 1990) (incorporated herein by reference). As illustrated in Fig. 5, R3616 contains a 1 Kbp deletion in both copies of the γ ,34.5 gene. In the 25 recombinant R4009 a stop codon was inserted in both copies of the γ ,34.5 gene. The γ ,34.5 genes in the recombinant R3616 were restored to yield the recombinant HSV-1(F)R.

Virus Infection Cells were generally exposed to the viruses for 2 h at 37°C at multiplicity of infection of 5 30 and then removed and replaced with the 199v media containing 1% calf serum. The infection continued at 37°C for a length of time as indicated for each experiment.

Cells were then either labeled for de novo protein synthesis or analysis of viral DNA and RNA.

³⁵S-methionine labeling At the indicated time post infection, 50uCi of ³⁵S-methionine (specific activity >1,000 Ci/mmol, Amersham Co., Downers Grove, IL) was added to 1ml of 199v media lacking methionine to cells in 6 well dishes. Labeling was continued for 1.5 hr, at which time cells were harvested. Preparation of cell extracts; separation of proteins by electrophoresis in denaturing polyacrylamide gels crosslinked with N,N' Diallyltartardiamide (Bio-Rad Laboratories, Richmond, CA); transfer of polypeptides to nitrocellulose sheets; autoradiography and immunoblot with antibodies have been described by Ackermann et al., J. Virol., 52: 108-118 (1984) (incorporated herein by reference).

Results

HSV-1 recombinant viruses lacking the γ ,34.5 gene induce the shut off protein synthesis in neuroblastoma cells. In the course of screening human cell lines derived from CNS tissues it was apparent that the SK-N SH neuroblastoma cell lines produced 100 fold less mutant viruses than the fully permissive Vero cells. It was also noted, as shown in Fig. 6, that the SK-N-SH neuroblastoma cells infected with R3616 or with R4009 exhibited reduced protein synthesis in cells harvested at 7 hours (left panel) and ceased to incorporate ³⁵S-methionine by 13 hours (right panel) post infection. The phenomenon was observed in SK-N-SH neuroblastoma cells only, and could be attributed specifically to the mutations in the γ ,34.5 gene inasmuch as restoration of the deleted sequences yielded a virus [HSV-1(F)R] which expressed viral proteins at 13 hour post infection (Fig. 6, right panel) and exhibited the parental, wild type phenotype.

The shut off of protein synthesis occurred after the expression of α genes. Viral genes form three major groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion. See Roizman and Sears, Fields' Virology, 2 ed., Fields et al., Eds, 1795-1841 (1990). The α genes do not require de novo protein synthesis for their expression, the β genes which are required for the synthesis of viral DNA require prior synthesis of functional α and β proteins and the onset of viral DNA synthesis. To determine the point at which expression of viral gene functions was terminated in SK-N-SH neuroblastoma cells infected with mutant viruses, infected cell lysates electrophoretically separated in denaturing polyacrylamide gels were transferred to a nitrocellulose sheet and probed with antibody to an α (α 27), a β (viral thymidine kinase) and two abundant γ proteins. Roller and Roizman (J. Virol., 65: 5873-5879 (1991) have shown that the latter were the products of U_{26.5} and of U₁₁ genes whose expression at optimal levels requires viral DNA synthesis. As shown in Fig. 7, the SK-N-SH neuroblastoma cells infected with the mutant viruses made normal amounts of α 27 protein (left panel), reduced amounts of the thymidine kinase (β) protein (middle panel), but no detectable γ proteins (left and right panels). In contrast, both the wild type and mutant viruses could not be differentiated with respect to their capacity to replicate or to direct the synthesis of their proteins in Vero cells (Fig. 7).

The signal for shut off of protein synthesis is linked to viral DNA synthesis. These experiments were designed to determine whether the shutoff of protein synthesis was linked to a gene whose expression was dependent on viral DNA synthesis. The results of a key experiment are shown in Fig. 8. Replicate SK-N-SH and Vero cell cultures were infected with HSV-1(F) and recombinant viruses and maintained in the presence or absence of inhibitory

concentrations of phosphonoacetate, a drug which blocks viral DNA synthesis. The cells were pulse labeled with S³⁵-methionine at 13 h post infection. The salient feature of the results were that protein synthesis in SK-N-SH cells 5 infected with either R3616 or R4009 was sustained for at least 13 h in the presence of Phosphonoacetate but not in its absence. These results indicated that the signal for cessation of protein synthesis in SK-N-SH neuroblastoma cells infected with mutant viruses was associated with 10 viral DNA synthesis or with a γ gene dependent on viral DNA synthesis for its expression.

Human neuroblastoma cells infected with the γ ,34.5- mutants synthesized viral DNA and accumulated late mRNA even though the shut off of protein synthesis precluded 15 accumulation of late proteins. The evidence presented above indicated that in SK-N-SH neuroblastoma cells an event associated with viral DNA synthesis triggered the shut off of protein synthesis and that the late (γ) viral proteins did not accumulate. We expected, therefore, 20 little or no accumulation of viral DNA and in the absence of viral DNA synthesis, little or no accumulation of late (γ) viral transcripts whose synthesis is dependent on viral DNA synthesis. To our surprise, the amounts of viral DNA recovered from SK-N-SH neuroblastoma cells 17 hours 25 post infection with mutant viruses were comparable to those obtained from wild type parent or repaired [(HSV-1)F]R viruses (Fig. 9, left panel). Furthermore, while the SK-N-SH neuroblastoma cells did not synthesize demonstrable amounts of U,11 protein, the amounts of U,11 gene 30 transcripts which accumulated in cells infected with mutant and wild type viruses were of similar magnitude (Fig. 9 right panel).

The significance of these results stems from three observations. First, in infected cells, protein synthesis 35 reflects a regulatory cascade; a protein synthesis is

replaced by β and later by γ protein synthesis. In all cell lines other than the SK-N-SH neuroblastoma cells infected with the $\gamma_{134.5}$ mutants and tested to date, a block in the synthesis of one group of proteins does not lead to
5 a cessation of total protein synthesis. For example, in cells treated with inhibitors of DNA synthesis like PAA, a subclass of γ proteins dependent for their synthesis on viral DNA synthesis is not made. However, in these cells, β protein synthesis continues beyond the time of their
10 synthesis in untreated infected cells. The striking observations made in the studies on SK-N-SH cells infected with the $\gamma_{134.5}$ null mutants are that (i) all protein synthesis ceased completely, (ii) viral DNA was made and (iii) late, γ mRNA exemplified by U₁₁ mRNA was made even
15 though protein synthesis ceased. These manifestations for viral replication have not been reported previously and are not characteristic of cells infected with wild type virus or any mutant virus infection of cells (e.g. Vero, HEp-2, baby hamster kidney, 143tk- and rabbit skin cell lines and
20 human embryonic lung cells strain) other than those described in this report.

Second, the function of the $\gamma_{134.5}$ gene is to overcome this block in protein synthesis in SK-N-SH cells since repair of the mutation restores the wild type phenotype.

25 Lastly, while the association of cessation of protein synthesis with the onset of viral DNA replication does not exclude the possibility that a product made after infection is responsible of the shut off, the data does support the hypothesis that the cessation of protein synthesis is
30 specifically caused by a known viral gene product interacting with the protein synthesizing machinery of the cell. For example, it has been well established that the product of the HSV-1 gene designated a vhs can shut off cellular protein synthesis after infection. vhs is a
35 structural protein of the virus and is introduced into

cells during infection. It destabilizes mRNA early in infection and its effects are not dependent on viral gene products made after infection. In the experiments set forth above, protein synthesis of wild type and mutant viruses could not be differentiated at 13 hours post infection in cells treated with Phosphonoacetate and hence the phenotype of mutant viruses cannot be attributed to the vhs gene product. This conclusion is reinforced by the observation that viral protein synthesis in SK-N-SH cells was not affected by increasing the multiplicity of infection with wild type virus to values as high as 100 pfu/cell (data not shown). A more likely source for the genetic information for the cessation of protein synthesis is the cell itself.

It has been reported that deprivation of growth factors from cells of neuronal origins results in programmed cell death, which manifests itself initially by the cessation of protein synthesis and subsequently by fragmentation of DNA. Apoptosis in lymphocytes is manifested by degradation of DNA. In the case of other herpes viruses, it has been shown that in B lymphocytes infected with the Epstein-Barr virus, the product of the viral LMP-1 gene induces the host gene Bcl-2 which precludes programmed lymphocyte death (Henderson et al, Cell 65: 1107-1191. Thus, it is apparent that the onset of viral DNA synthesis in neuronal cells triggers programmed cell death by cessation of protein synthesis and that HSV-1 γ 34.5 gene precludes this response.

The evolution of a HSV-1 gene which would preclude a response to a neuronal stress is not surprising. Infection of neurons, especially sensory neurons, is an essential feature of viral reproductive lifestyle which enables the HSV-1 to remain latent and to survive in human populations. If, as we propose, the function of γ 34.5 gene is to preclude cell death, the target of the gene would be

neurons rather than lymphocytes since HSV normally infects nerve cells.

The $\gamma_{134.5}$ gene has several unusual features. The gene lacks a conventional TATAA box or response elements 5 frequently associated with TATAA-less transcriptional units. The sequence which enables the expression of the gene is 12 bp long but repeated as many as 3 times in the wild type strain used in this laboratory. Various assays reported elsewhere indicate that the amounts of gene 10 products produced in cells of non neuronal derivation are smaller than those expressed by most viral genes and that the amounts of the protein made in the absence of viral DNA synthesis were smaller than those made in its presence. The gene is predicted to encode a protein of 263 amino 15 acids. It contains the triplet Ala-Thr-Pro repeated 10 time and accumulates in the cytoplasm. A recent note indicates that 63 amino acid residues near the carboxyl terminus of the $\gamma_{134.5}$ protein shares 83% identity with a mouse protein MyD116 found in a myeloid leukemic cell line 20 induced to differentiate by interleukin 6 (Lord et al., Nucleic Acid Res. 18: 2823, 1990). The function of MyD116 is unknown. The results presented above demonstrate that the product of the $\gamma_{134.5}$ gene, the protein ICP34.5, quite clearly enables sustained protein synthesis in SK-N-SH 25 neuroblastoma cells, and it is clear that the gene's expression is sufficient to preclude apoptosis.

The promoter-regulatory elements essential for the expression of $\gamma_{134.5}$ are contained within three elements of the α sequence, i.e. the direct repeats DR2 and DR4 and the 30 unique U_b sequences. Gel retardation assays failed to show binding of the product of the $\alpha 4$ gene encoding the major regulatory protein of the virus to any of the elements regulating expression of the $\gamma_{134.5}$ gene. In transient expression assays, the product of the $\alpha 4$ or of $\alpha 0$ genes 35 failed to transactivate a chimeric reporter gene consisting

of the coding sequences of the thymidine kinase gene fused to the 5' non-coding sequences of the γ ,34.5 gene. The reporter gene was induced, but to a relatively low level by co-transfection with plasmids containing both α 4 and α 0 genes. The plasmid encoding the α 27 gene had no effect on the expression of the chimeric reporter gene transfected alone although it reduced the induction of the chimeric gene by plasmids containing the α 0 and α 4 genes.

EXAMPLE 2 - TREATMENT OF PROGRAMMED CELL
10 DEATH (APOPTOSIS) WITH GENE THERAPY

In this example, γ ,34.5 gene therapy directed toward the prevention or treatment of apoptosis is described. For the purposes of this example mutated HSV-1 virus is proposed as a vector for introduction of the gene into 15 neuronal cells undergoing or about to undergo programmed cell death. It is also envisioned that this embodiment of the present invention could be practiced using alternative viral or phage vectors, including retroviral vectors and vaccinia viruses whose genome has been manipulated in 20 alternative ways so as to render the virus non-pathogenic. The methods for creating such a viral mutation are set forth in detail in U.S. Patent No. 4,769,331, incorporated herein by reference. Furthermore, it is also envisioned that this embodiment of the present invention could be 25 practiced using any gene whose expression is beneficial in gene therapy, and use of the non-HSV viruses would allow gene therapy in non-neural systems.

Herpes simplex virus has a natural tropism for human CNS tissue. Under wild type conditions, the virus is 30 capable of replicating and multiplying in the nervous system and is neurovirulent. The virus can also establish latent infection in the neurons and can be occasionally reactivated. To establish a vector system for delivery of genes into neurons, the proposed construct of an HSV vector 35 must satisfy the following criteria: 1. Such a vector

should have a natural tropism for CNS and brain tissue. 2. Such a vector should be non-pathogenic; that is, totally avirulent and not reactivatable to cause an infection. 3. Such a vector should consist of constitutive expression of 5 γ ;34.5 to prevent cell death in cells undergoing neurodegeneration. 4. Such a vector so proposed in 3 is suitable for additional foreign gene insertion for gene therapy.

Material and Methods

10 A HSV vector with a mutational lesion in the α 4 gene is constructed. The proposed virus will no longer be able to replicate, multiply and reactivate from latent infection in the CNS. The virus can, in the absence of α 4 gene, establish a latent infection in the neuron. This virus can 15 be obtained by co-transfection of viral DNA with plasmid containing a α 4 expressing cell line. α 4 expressing cell lines and the virus have been reported previously. DeLuca et al., J. Virol., 56: 558-570 (1985).

20 Additionally, such an HSV vector with γ ;34.5 gene under a constitutive expression promoter is also envisioned. This constitutive expression promoter can be the HSV LAT promoter, the LTR promoter of retrovirus or any other foreign promoter specific for neural gene expression. Such 25 a viral vector properly introduced is suitable for prevention of cell death in neuronal cells undergoing apoptosis.

30 Moreover, such an HSV vector with foreign genes inserted at a neutral location on the viral genome is suitable for delivery of foreign genes in target neurons and for CNS gene therapy. The procedures to generate the above recombinant viruses are those published by Post and Roizman (Cell, 25: 227, 1991) incorporated herein by reference. See also U.S. Patent No. 4,769,331, incorporated herein by reference.

In instances where use of the mutated HSV-1 virus is appropriate the virus can be combined with a pharmaceutically acceptable carrier such as buffered saline and injected at the site of peripheral nerve endings whose axons originate from neural cell bodies undergoing or about to undergo apoptosis. As will be recognized by those skilled in the medical arts the amount of virus administered will vary depending upon several factors, including the vector's ability to target the cells requiring treatment, the extent to which the gene is expressed in the target tissue, and the activity of the expressed protein, among others. An inoculum containing approximately 10^4 - 10^5 viruses in phosphate buffered saline or skim milk has produced successful results in mice.

Virus so injected is taken up into the peripheral nerve endings and is then transported via retrograde axonal transport to the neuronal cell bodies. In instances where such peripheral injection is not useful or appropriate, localized intraspinal or intraventricular injection, or direct microinjection of the virus could be utilized.

An appropriately altered non-HSV virus, one with a genome manipulated in such a way as to render the virus non-pathogenic, could be used in a similar manner. Direct microinjection or peripheral injection for delivery to the cell body via retrograde axonal transport are options for viral delivery. Finally, it should also be noted that a biological functional equivalent gene could be utilized for gene therapy in any vector described in this example.

EXAMPLE 3 - USE OF MULTI-POTENT NEURAL
30 CELL LINES TO DELIVER THE γ 34.5 GENE TO THE CNS

In addition to the viral vector delivery system to CNS and brain tissue, another vector system has been developed recently using cell lines passaged in vitro and engrafting these cells back to the animal. These procedures involve

taking cells of fetal or postnatal CNS origin, immortalizing and transforming them in vitro and transplanting the cells back into the mouse brain. These cells, after engraftment, follow the migration pattern and 5 environmental cue of normal brain cell development and differentiate in a nontumorigenic, cytoarchitecturally appropriate manner. This work has been exemplified in several articles notably Snyder et al., Cell, 68: 33-51, 1992 and Ranfranz et al., Cell, 66: 713-729, 1991.

10 Utilizing appropriately modified techniques, it is possible to introduce the γ ,34.5 gene alone or in combination with other genes of interest into the cells and engraft. Such a procedure allows the delivery of the genes to its natural site. Proper expression of the γ ,34.5 gene in these neurons 15 should result in prevention of cell death in neurodegeneration and preserving cells carrying foreign genes suitable for gene therapy.

Materials and Methods

20 **Propagation of Cerebellar Cell Lines** Cerebellar cell lines are generated as described by Ryder et al. (J. Neurobiol. 21: 356-375, 1990). Lines are grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco), 5% horse serum (Gibco), and 2mM glutamine on poly-L-lysine (PLL) (Sigma) (10 μ g/ml)-coated 25 tissue culture dishes (Corning). The lines are maintained in a standard humidified, 37°C, 5% CO₂-air incubator and are either fed weekly with one-half conditioned medium from confluent cultures and one-half fresh medium or split (1:10 or 1:20) weekly or semiweekly into fresh medium.

30 **Transduction of Cerebellar Progenitor Lines with γ ,34.5 Gene**

A recent 1:10 split of the cell line of interest is plated onto 60 mm tissue culture plates. Between 24 and 48 hr after plating, the cells are incubated with the

replication-incompetent retroviral vector BAG containing the -myc gene (10^6 - 10^7 colony-forming units [cfu]/ml) plus 8 μ g/ml polybrene for 1-4 hr for introduction of the γ ,34.5 gene alone or in combination with other suitable genes for 5 gene therapy, along with the neomycin G418 marker. Cells are then cultured in fresh feeding medium for approximately 3 days until they appear to have undergone at least two doublings. The cultures are then trypsinized and seeded at low density (50-5000 cells on a 100 mm tissue culture 10 dish). After approximately 2 weeks well-separated colonies are isolated by brief exposure to trypsin within plastic cloning cylinders. Colonies are plated in 24-well PLL-coated CoStar plates. At confluence, these cultures are 15 passaged to 60 mm tissue culture dishes and expended. A representative dish from each subclone is stained directly in the culture dish using X-gel histochemistry (see Price et al., 1967; Cepko, 1989a, 1989b). The percentage of blue cells is determined under the microscope. Subclones with the highest percentage of blue cells (ideally >90%; at 20 least >50%) are maintained, characterized, and used for transplantation.

Tests for Virus Transmission The presence of helper virus is assayed by measurement of reverse transcriptase activity in supernatants of cell lines as described by 25 Goff et al. (1981) and by testing the ability of supernatants to infect NIH 3T3 cells and generate G418-resistant colonies of X-gal⁺ colonies (detailed in Cepko, 1989a, 1989b). All cerebellar cell lines used for 30 transplantation are helper virus-free as judged by these methods.

Coculture of Neural Cell Lines with Primary Cerebellar Tissue Primary dissociated cultures of neonatal mouse cerebellum are prepared as in Ryder et al. (1990) and seeded at a density of 2×10^6 to 4×10^6 cells per PLL-coated eight-chamber Lab Tek glass or plastic slide 35

(Miles). After the cells settled (usually 24 hr), 10% of a nearly confluent 10 cm dish of the neural cell line of interest is seeded, following trypsinization, onto the slide. The coculture is re-fed every other day and grown in a 50% CO₂-air, humified incubator until 8 or 14 days of coculture.

Preparation of Cells Lines for Transplantation Cells from a nearly confluent but still actively growing dish of donor cells are washed twice with phosphate-buffered saline (PBS), trypsinized, gently triturated with a wide-bore pipette in serum-containing medium (to inactivate the trypsin), gently pelleted (1100 rp for 1 min in a clinical centrifuge), and resuspended in 5 ml of PBS. Washing by pelleting and resuspension of fresh PBS is repeated twice, with the cells finally resuspended in a reduced volume of PBS to yield a high cellular concentration (at least 1 x 10⁶ cells per μ l). Trypan blue (0.05% w/v) is added to localize the inoculum. The suspension is kept well triturated, albeit gently, and maintained on ice prior to transplantation to minimize clumping.

Injections into Postnatal Cerebellum Newborn CD-1 or CF-1 mice are cryoanesthetized, and the cerebellum is localized by transillumination of the head. Cells are administered either via a Hamilton 10 μ l syringe with a beveled 33-gauge needle or a drawn glass micropipette with a 0.75 mm inner diameter and 1.0 mm outer diameter generated from borosilicate capillary tubing (FHC, Brunswick, ME) by a Flaming Brown Micropipette Puller (Model p-87, Sutter Instruments) using the following parameters: heat 750, pull 0, velocity 60, time 0. Best results are achieved with the glass micropipette. The tip is inserted through the skin and skull into each hemisphere and vermis of the cerebellum where the cellular suspension was injected (usually 1-2 μ l per injection). Typically, the following situation should exist: 1 x 10⁷ cells per ml

of suspension; $\times 10^6$ to 2×10^6 cells per injection; one injection in each cerebellar hemisphere and in the vermis. Importantly, the cellular suspension, maintained on ice throughout, is gently triturated prior to each injection in 5 order to diminish clumping and to keep cells suspended.

The injection of BAG virus was performed as described for the cell suspension. The BAG virus stock (8×10^7 G418-resistant cfu/ml) contained, in addition to trypan blue, polybrene at 8 µg/ml.

10 EXAMPLE 4 - TREATMENT OF PROGRAMMED CELL DEATH
(APOPTOSIS) WITH ICP34.5

As an alternative to the gene therapy methods described for exemplary purposes in Examples 2 and 3, neuronal cells undergoing or about to undergo programmed 15 cell death can also be treated with the protein expressed by the γ ,34.5 gene, i.e. ICP34.5. Alternatively, a biological functional equivalent protein could be used in such treatment.

For example, ICP34.5 is isolated from cells expressing 20 the protein and purified using conventional chromatography purification and immunoaffinity purification methods described by Ackerman et al. (J. Virol. **58**: 843-850, 1986, incorporated herein by reference). The purified protein is next combined with a pharmaceutically appropriate carrier, 25 such as buffered saline or purified distilled water. For administration, the pharmaceutical composition can be injected in one of several ways, as appropriate: (i) intraspinal injection; (ii) intraventricular injection; (iii) direct injection into the area containing the neurons 30 undergoing or about to undergo programmed cell death or any other appropriate method of administration understood by those skilled in the art. Such treatment would be particularly appropriate in the surgical repair of severed peripheral nerves, and the use of proteins as therapeutic

agents is well within the current level of skill in the medical arts in light of the present specification.

**EXAMPLE 5 - ASSAYS FOR CANDIDATE SUBSTANCES FOR
PREVENTION OF PROGRAMMED CELL DEATH (APOPTOSIS)**

- 5 The γ ,34.5 gene of herpes simplex virus enables the virus to replicate, multiply and spread in the central nervous system and the brain so that the virus is neurovirulent to the host. Recombinant virus lacking the gene lost this ability to penetrate the CNS of the host and 10 become totally avirulent. In examining the nature of this avirulent phenotype in culture, the mutant virus lacking the gene exhibited a total translation shutoff phenotype characteristic of programmed cell death. This mechanism of programmed cell death afforded by the host cell greatly 15 reduced the ability of the virus to multiply and spread. The function of γ ,34.5 in the virus therefore is to inactivate the programmed death of the cell (anti-apoptosis) thereby restoring translation and enabling the virus to replicate to full potential in the host.
- 20 This anti-apoptotic effect of γ ,34.5 was further examined and its ability to protect neural cells from other environmental stresses which lead to apoptosis was discovered. These environmental stresses include UV, nerve grown factor deprivation and neuronal cell differentiation. 25 This Example describes the use of the γ ,34.5 gene and its protective function to screen for pharmaceutical agents and drugs that mimic the *in vivo* function of γ ,34.5 to prevent neurodegeneration. Such a screening procedure constitutes construction of cell lines expressing γ ,34.5 and a null cell 30 line without the gene and measurement of the viability of the cells after stress treatment by induction of a reporter gene. This can be a host gene promoter tagged by a fluorescence indicator or any other easily assayable marker to signal viability.

Materials and Methods

A test neuroblastoma cell line is established constitutively expressing $\gamma_{134.5}$ and containing a fluorescence tagged (e.g., the α sequence promoter fused to lacZ) cellular gene, or any tag that provides the easily assayable marker to signal viability. A neuroblastoma null cell line consisting of α -lacZ indicator gene and the same host indicator gene is also established, along with a Vero cell line consisting of α -lacZ indicator gene and the same host indicator gene. Environmental stresses are then applied that normally would (1) trigger the α sequence promoter activation; (2) trigger the protection afforded by $\gamma_{134.5}$ as signaled by viability after stress treatment; and (3) trigger cell programmed death in the absence of $\gamma_{134.5}$. Candidate substances of pharmaceutically appropriate drugs and agents can be tested in such an assay. The proposed scheme of the assay for scoring of positive candidates is shown in outline form in Table 2.

TABLE 2

EXPERIMENTAL FLOW CHART: ASSAY FOR CANDIDATE SUBSTANCE ABLE TO PREVENT PROGRAMMED CELL DEATH

	<u>CELL LINES</u>	<u>ACTION</u>	<u>EXPECTATION</u>
20	A. neuroblastoma cells constitutively express $\gamma_{134.5}$ and a second inducible promoter-indicator gene	stress followed by induction of second promoter	viability as measured by induction of a reporter gene hours after stress
25	B. neuroblastoma cells expressing α -lacZ and a second inducible promoter	stress followed by induction of second promoter	1. apoptosis related stress: α -lacZ induced; second promoter <u>not</u> induced 2. toxicity: no induction of α -lacZ and the second inducible promoter
30			
35			

- C. vero cells
expressing α -lacZ
and a second inducible
promoter gene
- 5 stress followed
by induction
1. α -lacZ not
induced
2. toxicity
factor exclu-
ded, determined
from expression
of inducible
second promoter

10 EXAMPLE 6 - ASSAY FOR CANDIDATE SUBSTANCES FOR
ACTIVATION OF PROGRAMMED CELL DEATH
(APOPTOSIS) FOR TREATMENT OF CANCER OR
TUMOROGENIC DISEASES AND FOR SUPPRESSION OF HSV INFECTION

In order to induce cell death in tumor cells, it is
15 desirable to block the expression of the anti-apoptosis
gene or the activity of the protein expressed by the gene.
As such, it is desirable to develop procedures that will
allow screening for candidate substances which trigger cell
death in tumor cells. In addition, since expression of the
20 γ ,34.5 gene of HSV-1 has been shown to prevent apoptosis in
neuronal cells so that the virus can replicate, multiply
and spread in the CNS (that is, so that the virus can
become neurovirulent), a substance capable of blocking
 γ ,34.5 expression or inhibiting the action of ICP34.5 can be
25 expected to suppress HSV neurovirulence (or the virulence of
other viruses relying on a similar mechanism) by allowing
apoptosis to occur in infected neurons.

It has been found that the protection afforded by
 γ ,34.5 can be extended to protect other cells from
30 environmental stresses, and indeed the gene has a
generalized anti-apoptotic effect. The promoter for the
gene γ ,34.5 lies in the α sequence of HSV and, at time of
stress, the promoter is activated. It can be assumed that
the α sequence promoter contains apoptosis responsive
35 elements and cellular factors (transcription factors in
particular) that mediate the expression of anti-apoptosis
gene are apoptotic in nature. These cellular factors are
therefore the targets of the present assay to screen for
drugs or agents that would inactivate their ability to

induce the anti-apoptosis gene. The assay involves the use of the α sequence promoter and its inducibility by conditions which induce apoptosis as an indicator assay which screens for therapeutic agents and drugs capable of blocking the expression of the anti-apoptosis gene and therefore allow the cell to die of programmed cell death.

A test plasmid construct bearing the α sequence and coding sequence up to the 28th amino acid of $\gamma_134.5$ is fused to the lacZ reporter gene or any other readily assayable reporter gene. The construct is introduced into a neuroblastoma or PC12 cell line by G418 selection and a clonal and continuous cell line for screening purposes is established. A control plasmid construct bearing an HSV late promoter, a promoter which would normally not be expressed in cell lines and which further would not be induced to express by apoptosis-inducing stress is fused to the same indicator gene. This construct is also introduced into a continuous clonal cell line and serves as a control for the test cell line. Environmental stresses that trigger the α sequence promoter activation and that cause programmed cell death are then defined. These conditions include UV injury, virus infection, nerve growth factor deprivation, and the influence of antibodies on cell surface receptors, among others. Candidate substances or pharmaceutically appropriate drugs and agents are then tested in assays for their ability to block the α sequence promoter activation at time of stress.

The assay of the present invention allows the screening and identification of pharmaceutically appropriate drugs and agents targeted at various cellular factors that induce the expression of anti-apoptosis gene. By inactivating essential cellular factors, these agents should be able to allow cell programmed death to occur. Such positive candidates would then be appropriately administered (via intravenous, intrathecal, or direct injection, or via oral administration) in order to induce programmed cell death in tumor cells, in neurons infected

with the herpes virus, or in cells infected with a virus the virulence of which is dependent upon an anti-apoptotic effect. The use of proteins and other chemotherapeutic substances in antitumor therapy is well known in the art, 5 and therefore, it is considered that the use and dosages of candidate substances for treatment of tumorogenic diseases (e.g., cancer) or herpes infection is well within the skill of the present state of the medical arts in light of the present specification. See U.S. Pat. Nos. 4,457,916; 10 4,529,594; 4,447,355; and 4,477,245, all incorporated herein by reference. These positive candidates can also be used to identify intermediates in the pathways leading to cell programmed death.

Materials and Methods

15 W5 cell lines are established in 96 well culture dishes coated with collagen. Control cell lines containing the promoter fusion element are also established in such 96 well dishes. The test candidate substances are added to the medium in individual wells containing both the test and 20 control cell lines set up in 96 well dishes. The cells are then briefly exposed to UV or other stresses. 8 hr post stress induction, cells are washed with PBS-A twice, and fixed with 0.5 ml containing 2% (v/v) formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature. The 25 cells are rinsed again with PBS and then stained with 2ml 5mM potassium ferrocyanide, 5mM potassium ferrocyanide, 2mM MgCl₂, and 1mg/ml X-gal (diluted from a 40mg/ml stock solution in dimethyl sulfoxide) in PBS. Cells expressing β-Galactosidase were stained blue after incubation at 37°C 30 for 2-3 days.

Results

The construct described above with the lacZ reporter gene was introduced into PC12 cell line. A new cell line W5 was clonally established by G418 selection. The W5 cell line was then tested for activation of the a sequence 35 promoter under suboptimal conditions names in 3 above. The results are: (a) The above cells, when exposed briefly to

UV for 2 minutes, turn blue upon staining the fixed cells with X-gal at 6-10 hr post UV exposure. (b) The above cells, when exposed to HSV-1(F) virus at multiplicity of infection of 5, also turn blue upon staining at 8 hr post 5 infection. (c) The above cells turn light blue when nerve growth factor (rat, 7S) is introduced into the medium to allow differentiation processes. (d) The cells turn darker blue when Nerve Growth Factor is removed from the medium after differentiation is complete and the cells have become 10 dependent on nerve growth factor for survival. (e) Little or no difference in color development is seen in cells starved for serum (0% fetal bovine serum) and those fully supplied in 10% fetal bovine serum. (f) The above experiments are repeated with control promoter fusion 15 elements to control for the true inhibition of anti-apoptosis gene expression rather than toxicity-induced cell death. By this procedure, the positive candidates that can induce cell death in cells will therefore render the following phenotypes: (i) Introduction of stress to the 20 test cell line in the absence of this substance will give rise to blue colored cells. (ii) Introduction of stress to the test cell line in the presence of same substance will give rise to white cells.

(iii) Introduction of stress to control cell lines with our 25 without this putative substance will have no effect on the color of cells.

The present invention has been disclosed in terms of specific embodiments which are believed by the inventors to be the best modes for carrying out the invention. However, 30 in light of the disclosure hereby provided, those of skill in the various arts will recognize that modifications can be made without departing from the intended scope of the invention. The exemplary embodiments set forth herein and all other modifications and embodiments are intended to be 35 within the scope and spirit of the present invention and the appended claims.

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Chou, Joany
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTTAAAGTCGGGGCGGC

17

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCAGCCCCGGC CCCCCGCGGC CGAGACGAGC GAGTTAGACA GGCAAGCACT ACTCGCCTCT	60
GCACGGCACAT GCTTGCTGT CAAACTCTAC CACCCCGGCA CGCTCTCTGT CTCCATGGCC	120
CGCCGCCGCC GCC	133

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 291 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCGCGGCCCG CCGCCGCCCC CGGCCGCCCG GGCCCACGGG CGCCGTCCCA ACCGCACAGT	60
CCCAGGTAAC CTCCACGCCCG AACTCGGAAC CCGCGGTCAG GAGCGCGCCC GCGGCCGCC	120
CGCCGCCGCC CCCCGCCAGT GGGCCCCCGC CTTCTTGTTC GCTGCTGCTG CGCCAGTGGC	180
TCCACGTTCC CGAGTCCGCG TCCGACGACG ACGATGACGA CGACTGGCCG GACAGCCCC	240
CGCCCGAGCC GGCGCCAGAG GCCCGGCCCA CCGCCGCCGC CCCCCGCC C	291

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 595 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACCGCCCCGGC GCGGGCCCGG GGGGCGGGGC TAACCCCTCC CACCCCCCT CACGCCCTT	60
CCGCCTTCCG CCGCGCCTCG CCCTCCGCCT GCGCGTCACC GCAGAGCACC TGGCGCGCCT	120
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGGAGCCC CCCGCGACCC CCGCGACCCC	180
CGCGACCCCC GCGACCCCCG CGACCCCCGC GACCCCCGCG ACCCCCCGCGA CCCCCCGCGAC	240
CCCCCGCGACC CCCCGCGGGG TGCCTTCTC GCCCCACGTC CGGGTGCGCC ACCTGGTGGT	300
CTGGGGCTCG GCGGCCCCGC TGGCGCGCCG CGGCTCGTGG GCGCGCGAGC GGGCGACCG	360
GGCTCGGTTG CGGCGCGGGG TGGCGGAGGC CGAGGGGGTC ATCGGGCCGT GCCTGGGGCC	420
CGAGGGCCGT GCGCGGGCCC TGGCCCGCGG AGCGGGCCCG GCGAACTCGG TCTAACGTTA	480
CACCCGAGGC GGCCTGGGTC TTCCGCGGAG CTCCCGGGAG CTCCGACCCA AGCCGCTCTC	540
CGGAGAGACG ATGGCAGGAG CCGCGCATAT ATACGCTGGG AGCGGGCCCG CCCCC	595

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 291 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGGCAGGGCC CGCCCTCGGA GGGCGGGACT GGCCAATCGG CGGCCGCCAG CGCGGCGGGG	60
CCCGGCCAAC CAGCGTCCGC CGAGTCTTCG GGGCCCGGCC CACTGGCGG GAGTTACCGC	120
CCAGTGGGCC GGGCCGCCCA CTTCCCGGTA TGGTAATTAA AAACTTACAA GAGGCCTTGT	180
TCCGCTTCCC GGTATGGTAA TTAGAAACTC ATTAATGGC GGCCCCGGCC GCCCTTCCCG	240
CTTCCGGCAA TTCCCCGGC CCTTAATGGG CAACCCCGGT ATTCCCCGCC T	291

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 150 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTAAAGCGG TGGCGGCGGG CAGCCCGGGC CCCCCGCCGA GACTAGCGAG TTAGACAGGC	60
AAGCACTACT CGCCTCTGCA CGCACATGCT TGCCTGTCAA ACTCTACCAC CCCGGCACGC	120
TCTCTGTCTC CATGGCCCGC CGCCGCCGCC	150

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 503 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCGGGCCCC CCGCCGCCCC CGGCCGCCCC GGCCCACGGG CGCCGTCCCA ACCGCACAGT	60
CCCAGGTAAC CTCCACGGCCC AACTCGGAAC CGCGGGTCAG GAGCGCGCCC GCGGCCGCCC	120
CGCCGCCGCC CCCCGCCGGT GGGCCCCCGC CTTCTTGTTC GCTGCTGCTG CGCCAGTGGC	180
TCCACGTTCC CGAGTCCGCG TCCGACGACG ACGATGACGA CGACTGGCCG GACAGCCCCC	240
CGCCCGAGTC GGCGCCAGAG GCCCGGCCCCA CGGCCGCCGC CCCCCGGCCCC CGGGGGCCCC	300
ACCGCCCGGC GTGGGCCCCGG GGGGCGGGGC TGACCCCTCC CACCCCCCCT CGCCGCCCTT	360
CCGCCTTCCG CCGCGCCTCG CCCTCCGCCT GCGCGTCACC GCGGAGCACC TGGCGCGCCT	420
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGGAGCCC CCCGCGACCC CGCGACCCC	480
CGCGACCCCCC GCGACCCCCCG CGA	503

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 368 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCCCGCGAC	CCCCGCGCGG	GTGCGCTTCT	CGCCCCACGT	CGGGGTGCGC	CACCTGGTGG	60
TCTGGGCCTC	GGCCGCCCGC	CTGGCGCGCC	GCGGCTCGTG	GGCCCGCGAG	CGGGCCGACC	120
GGGCTCGGTT	CCGGCGCCGG	GTGGCGGAGG	CCGAGGCGGT	CATCGGGCCG	TGCCTGGGGC	180
CCGAGGCCCG	TGCCCGGGCC	CTGGCCCGCG	GAGCCGGCCC	GGCGAACTCG	GTCTAACGTT	240
ACACCCGAGG	CGGCCTGGGT	CTTCCGCGGA	GCTCCCGGGA	GCTCCGCACC	AAGCCGCTCT	300
CCGGAGAGAC	GATGGCAGGA	GCCGCGATA	TATACGTTG	GAGCCAGCCC	GCCCTCACAG	360
GGCGGGCC						368

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 271 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGCGGGACT	GGCCAATCGG	CGGCCGCCAG	CGCGCGGGGG	CCCGGCCAAC	CAGCGTCCGC	60
CGAGTCTTCG	GGGCCCCGCC	CATTGGCGGG	GAGTTACCGC	CCAATGGGCC	GGGCGGCCCA	120
CTTCCCGGTA	TGGTAATTAA	AAACTTGCAA	GAGGCCTTGT	TCCGCTTCCC	GGTATGGTAA	180
TTAGAAACTC	ATTAATGGGC	GGCCCCGGCC	GCCCTTCCCG	CTTCCGGCAA	TTCCCGCGGC	240
CCTTAATGGG	CAACCCCGGT	ATTCCCCGCC	T			271

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 11 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTAAAGTCA	C					11
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(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 256 bas pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCGGGGGGC AGCCCCCCC CGGCCGAGAC TAGCGAGTTA GACAGGCAAG CACTACTCGC	60
CTCTGCACCC ACATGCTTGC CTGTCAAACCT CTACCACCCC GGCACGCTCT CTGTCTCCAT	120
GGCCCGCCGC CGCCGCCGCC ATCGCGGCCCG CCGCCGCCCG CCGCCGCCCG GGCCCACGGG	180
CGCGGTCCCC ACCGCACAGT CCCAGGTAAC CTCCACGCC AACTCGGAAC CCGTGGTCAG	240
GAGCGGCCGC CGGGCC	256

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 154 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTGGGGCCC CGCCTTCTTG TTCGCTGCTG CTGCGCCAGT GGCTCCACGT TCCCGAGTCC	60
GCGTCCGACG ACGACGATGA CGACGACTGG CCGGACAGCC CCCCCGCCGA GCCGGCGCCA	120
GAGGCCCCGGC CCACCGCCGC CGCCCCCCGC CCCC	154

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 212 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACCGCCCCGGC GCAGGGCCCGG GGGGCGGGGC TAACCCCTCC CACCCCCCTT CACGCCCTT	60
CCGCCTTCCG CCGCGCCTCG CCCTCCGCCT GCGCGTCACC GCGGAGCACC TGGCGCGCCT	120
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGAAGCCC CCCGCGACCC CCGCGACCC	180

CGCGACCCCC CGCACCCCCG CGACCCCCGC GA

212

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 356 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCCCCGCGAC	CCCCGCGCGG	GTGCGCTTCT	CGCCCCACGT	CCGGGTGCAG	CACCTGGTGG	60
TCTGGGCCTC	GGCCGCCCGC	CTGGCGCGCC	GCGGCTCGTG	GGCCCGCGAG	CGGGCCGACC	120
GGGCTCGGTT	CCGGCGCCGG	GTGGCGGAGG	CCGAGGCGGT	CATCGGGCCG	TGCCCTGGGGC	180
CCGAGGCCCG	TGCCCAGGCC	CTGGCCCGCC	GAGCCGGCCC	GGCGAACTCG	GTCTAACGTT	240
ACACCCGAGG	CGGCCTGGGT	CTTCCGCGGA	GCTCCCGGGA	GCTCCACACC	AAGCCGCTCT	300
CCGGAGAGAC	GATGGCAGGA	GCCGCGCATA	TATACGCTGG	GAGCCGGCCC	GCCCCC	356

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 291 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGGCCGGGCC	CGCCCTCGGA	GGGCCGGACT	GGCCAATCGG	CGGCCGCCAG	CGCCGGCGGGG	60
CCCGGCCAAC	CAGCGTCCGC	CGAGTCGTG	GGGCCCGGCC	CACTGGGCCG	TAACTCCCGC	120
CCAGTGGGCC	GGGCCGCCCA	CTTCCCGGTA	TGGTAATTAA	AAACTTGCAA	GAGGCCTTGT	180
TCCGCTTCCC	GGTATGGTAA	TTAGAAACTC	ATTAATGGGC	GGCCCGGGCC	GCCCTTCCCG	240
CTTCCGGCAA	TTCCCGCGGC	CCTTAATGGG	CAACCCCGGT	ATTCCCCGCC	T	291

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid

64

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTTAAAGCGC

10

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 431 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCGGGCGGGC AGCCCCCCCCG CGGCCGAGAC TAGCGAGTTA GACAGGCAAG CACTACTCGC	60
CTCTGCACGC ACATGCTTGC CTGTCAAACT CTACCACCCC GGCACGCTCT CTGTCTCCAT	120
GGCCCCGCCGC CGCCGCCGCC ATCCGCGGCC CCGCCGCCCC CGGCCGCCCG GGCCCACGGG	180
CGCGGTCCCA ACCGCACAGT CCCAGGTAAC CTCCACGCC AACTCGGAAC CCGTGGTCAG	240
GAGCGCGCCC CGGGCCGCC CGCCGCCGCC CCCCAGCCGT GGGCCCCCGC CTTCTTGTTC	300
GCTGCTGCTG CGGCAGTGGC TCCAGGTTCC GGAGTCCGCG TCCGACGACG ACGATGACGA	360
CGACTGGCCG GACAGCCCCC CGCCCGAGCC GGCGCCAGAG GCCCGGCCCA CCGCCGCCGC	420
CCCCCGCCCC C	431

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 212 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACCGCCCCGGC GCGGGCCAG GGGGCGGGGC TGACCCCTCC CACCCCCCT CACGCCCTT	60
CCGCCTTCCG CCGCGCCTCG CCCTCCGCCT GCGCGTCACC GCAGAGCACC TGGCGCGCCT	120
GCGCCTGCGA CGCGCGGGCG GGGAGGGGGC GCCGGAGCCC CCCGCGACCC CCGCGACCCC	180
CGCGACCCCCC GCGACCCCCG CGACCCCCGC GA	212

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 356 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCCCCGCGAC	CCCCGCGCGG	GTGCGCTTCT	CGCCCCACGT	CCGGGTGCGC	CACCTGGTGG	60
TCTGGGCCTC	GGCCGCCCGC	CTGGCGCGCC	GCGGCTCGTG	GGCCCGCGAG	CGGGCCGACC	120
GGGCTCGGTT	CCGGCGCCGG	GTGGCGGAGG	CCGAGGCGGT	CATCGGGCCG	TGCCTGGGCC	180
CCAAGGCCG	CGCCCCGGCC	CTGGCCCGCG	GAGCCGGCCC	GGCGAACTCG	GTCTAACGTT	240
ACACCCGAGG	CGGGCTGGGT	CTTCCGCGGA	GCTCCCGGGA	GCTCCACACC	AAGCCGCTCT	300
CCGGAGAGAC	GATGGCAGGA	GCCGCGATA	TATACGCTGG	GAGCCGGCCC	CCCCCC	356

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 291 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAGGCCGGGCC	CGCCCTCGGA	GGGCAGGACT	GGCCAATCGG	CGGCCGCCAG	CGCGGCGGGG	60
CCCGGCCAAC	CAGCGTCCGC	CGAGTCGTG	GGGCCCGGCC	CACTGGCGG	TAACTCCCAC	120
CCAGTGGGCC	GGGCCGCCCA	CTTCCCGGTA	TGGTAATTAA	AAACTTGCAA	GAGGCCTTGT	180
TCCGCTTCCC	GGTATGGTAA	TTAGAAACTC	ATTAATGGC	GGCCCCGGCC	GCCCTTCCCG	240
CTTCCGGCAA	TTCCCGCGGC	CCTTAATGGG	CAACCCCGGT	ATTCCCCGCC	T	291

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTAACCTAGA	CTAGTCTAGC		20
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(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATCTGATCA GATCGCATTG

20

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 58 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCCGGACATG GAACGAGTAC GACGACGCAG CCGACGCCGC CGGCGACCCGG GCCCCGGG

58

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 51 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTGCTCATGC TGCTGCGTCG GCTGCGCGG CCGCTGGCCC GGGGCCCGTA C

51

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Ala Arg Arg Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 258 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

His Arg Gly Pro Arg Arg Pro Arg Pro Pro Gly Pro Thr Gly Ala Val
 1 5 10 15
 Pro Thr Ala Gln Ser Gln Val Thr Ser Thr Pro Asn Ser Glu Pro Ala
 20 25 30
 Val Arg Ser Ala Pro Ala Ala Pro Pro Pro Pro Ala Ser Gly
 35 40 45
 Pro Pro Pro Ser Cys Ser Leu Leu Leu Arg Gln Trp Leu His Val Pro
 50 55 60
 Ala Glu Ser Ala Ser Asp Asp Asp Asp Asp Asp Trp Pro Asp Ser
 65 70 75 80
 Pro Pro Pro Glu Pro Ala Pro Glu Ala Arg Pro Thr Ala Ala Pro
 85 90 95
 Arg Pro Arg Ser Pro Pro Pro Gly Ala Gly Pro Gly Gly Ala Asn
 100 105 110
 Pro Ser His Pro Pro Ser Arg Pro Phe Arg Leu Pro Pro Arg Leu Ala
 115 120 125
 Leu Arg Leu Arg Val Thr Ala Glu His Leu Ala Arg Leu Arg Leu Arg
 130 135 140
 Arg Ala Gly Gly Glu Gly Ala Pro Glu Pro Pro Ala Thr Pro Ala Thr
 145 150 155 160
 Pro Ala Thr Pro
 165 170 175
 Ala Thr Pro Ala Thr Pro Ala Arg Val Arg Phe Ser Pro
 180 185 190
 His Val Arg Val Arg His Leu Val Val Trp Ala Ser Ala Ala Arg Leu
 195 200 205
 Ala Arg Arg Gly Ser Trp Ala Arg Glu Arg Ala Asp Arg Ala Arg Phe
 210 215 220
 Arg Arg Arg Val Ala Glu Ala Glu Ala Val Ile Gly Pro Cys Leu Gly
 225 230 235 240
 Pro Glu Ala Arg Ala Arg Ala Leu Ala Arg Gly Ala Gly Pro Ala Asn
 245 250 255
 Ser Val

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Ala Arg Arg Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

His Arg Gly Pro Arg Arg Pro Arg Pro Pro Gly Pro Thr Gly Ala Val
1 5 10 15

Pro Thr Ala Gln Ser Gln Val Thr Ser Thr Pro Asn Ser Glu Pro Ala
20 25 30

Val Arg Ser Ala Pro Ala Ala Ala Pro Pro Pro Pro Ala Gly Gly
35 40 45

Pro Pro Pro Ser Cys Ser Leu Leu Leu Arg Gln Trp Leu His Val Pro
50 55 60

Glu Ser Ala Ser Asp Asp Asp Asp Asp Asp Trp Pro Asp Ser Pro
65 70 75 80

Pro Pro Glu Ser Ala Pro Glu Ala Arg Pro Thr Ala Ala Ala Pro Arg
85 90 95

Pro Pro Gly Pro His Arg Pro Ala Trp Ala Arg Gly Ala Gly Leu Thr
100 105 110

Pro Pro Thr Pro Pro Arg Ala Pro Ser Ala Phe Arg Arg Ala Ser Pro
115 120 125

Ser Ala Cys Ala Ser Pro Arg Ser Thr Trp Arg Ala Cys Ala Cys Asp
130 135 140

Ala Arg Ala Gly Arg Gly Arg Arg Ser Pro Pro Arg Pro Pro Arg Pro
145 150 155 160

Pro Arg Pro Pro Arg Pro Pro Arg Pro
165

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 180 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: singl
- (D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Pro	Arg	Gly	Cys	Ala	Ser	Arg	Pro	Thr	Ser	Gly	Cys	Ala	Thr	Trp	Trp
1				5					10					15	
Ser	Gly	Pro	Arg	Pro	Pro	Ala	Trp	Arg	Ala	Ala	Ala	Arg	Gly	Pro	Ala
		20						25					30		
Ser	Gly	Pro	Thr	Gly	Leu	Gly	Ser	Gly	Ala	Gly	Trp	Arg	Arg	Pro	Arg
		35					40				45				
Arg	Ser	Ser	Gly	Arg	Ala	Trp	Gly	Pro	Arg	Pro	Val	Pro	Gly	Pro	Trp
	50				55				60						
Pro	Ala	Glu	Pro	Ala	Arg	Arg	Thr	Arg	Ser	Asn	Val	Thr	Pro	Glu	Ala
	65				70				75			80			
Ala	Trp	Val	Phe	Arg	Gly	Ala	Pro	Gly	Ser	Ser	Ala	Pro	Ser	Arg	Ser
		85						90				95			
Pro	Glu	Arg	Arg	Trp	Gln	Glu	Pro	Arg	Ile	Tyr	Thr	Leu	Gly	Ala	Ser
	100						105					110			
Pro	Pro	Ser	Gln	Gly	Gly	Pro	Pro	Arg	Gly	Arg	Asp	Trp	Pro	Ile	Gly
	115					120				125					
Gly	Arg	Gln	Arg	Gly	Gly	Ala	Arg	Pro	Thr	Ser	Val	Arg	Arg	Val	Phe
	130					135				140					
Gly	Ala	Arg	Pro	Ile	Gly	Arg	Glu	Leu	Pro	Pro	Asn	Gly	Pro	Gly	Arg
	145				150				155			160			
Pro	Leu	Pro	Gly	Met	Val	Ile	Lys	Asn	Leu	Gln	Glu	Ala	Leu	Phe	Arg
		165						170				175			
Phe	Pro	Val	Trp												
		180													

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 46 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

72

Met Ala Arg Arg Arg Arg Arg His Arg Gly Pro Arg Arg Pro Arg Pro
1 5 10 15

Pro Gly Pro Thr Gly Ala Val Pro Thr Ala Gln Ser Gln Val Thr Ser
20 25 30

Thr Pro Asn Ser Glu Pro Val Val Arg Ser Ala Pro Ala Ala
35 40 45

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 126 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Gly Gly Pro Pro Pro Ser Cys Ser Leu Leu Leu Arg Gln Trp Leu His
1 5 10 15

Val Pro Glu Ser Ala Ser Asp Asp Asp Asp Asp Asp Asp Trp Pro Asp
20 25 30

Ser Pro Pro Pro Glu Pro Ala Pro Glu Ala Arg Pro Thr Ala Ala Ala
35 40 45

Pro Arg Pro Arg Ser Pro Pro Gly Ala Gly Pro Gly Gly Ala
50 55 60

Asn Pro Ser His Pro Pro Ser Arg Pro Phe Arg Leu Pro Pro Arg Leu
65 70 75 80

Ala Leu Arg Leu Arg Val Thr Ala Glu His Leu Ala Arg Leu Arg Leu
85 90 95

Arg Arg Ala Gly Gly Glu Gly Ala Pro Lys Pro Pro Ala Thr Pro Ala
100 105 110

Thr Pro Ala Thr Pro Ala Thr Pro Ala Thr Pro Ala Thr Pro
115 120 125

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 73 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Ala Arg Val Arg Phe Ser Pro His Val Arg Val Arg His Leu Val Val
1 5 10 15

Trp Ala Ser Ala Ala Arg Leu Ala Arg Arg Gly Ser Trp Ala Arg Glu
20 25 30

Arg Ala Asp Arg Ala Arg Phe Arg Arg Arg Val Ala Glu Ala Glu Ala
35 40 45

Val Ile Gly Pro Cys Leu Gly Pro Glu Ala Arg Ala Arg Ala Leu Ala
50 55 60

Arg Gly Ala Gly Pro Ala Asn Ser Val
65 70

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 179 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met	Ala	Arg	Arg	Arg	Arg	Arg	His	Arg	Gly	Pro	Arg	Arg	Pro	Arg	Pro
1							5			10				15	
Pro	Gly	Pro	Thr	Gly	Ala	Val	Pro	Thr	Ala	Gln	Ser	Gln	Val	Thr	Ser
			20					25					30		
Thr	Pro	Asn	Ser	Glu	Pro	Val	Val	Arg	Ser	Ala	Pro	Ala	Ala	Pro	
			35				40				45				
Pro	Pro	Pro	Pro	Ala	Gly	Gly	Pro	Pro	Pro	Ser	Cys	Ser	Leu	Leu	Leu
				50		55					60				
Arg	Gln	Trp	Leu	Gln	Val	Pro	Glu	Ser	Ala	Ser	Asp	Asp	Asp	Asp	Asp
				65		70				75			80		
Asp	Asp	Trp	Pro	Asp	Ser	Pro	Pro	Glu	Pro	Ala	Pro	Glu	Ala	Arg	
				85			90				95				
Pro	Thr	Ala	Ala	Ala	Pro	Arg	Pro	Ser	Pro	Pro	Pro	Gly	Ala	Gly	
				100		105					110				
Pro	Gly	Gly	Gly	Ala	Asp	Pro	Ser	His	Pro	Pro	Ser	Arg	Pro	Phe	Arg
				115		120					125				
Leu	Pro	Pro	Arg	Leu	Ala	Leu	Arg	Leu	Arg	Val	Thr	Ala	Glu	His	Leu
				130		135				140					
Ala	Arg	Leu	Arg	Leu	Arg	Arg	Ala	Gly	Gly	Glu	Gly	Ala	Pro	Glu	Pro
				145		150				155			160		
Pro	Ala	Thr	Pro												
				165			170				175				
Ala	Thr	Pro													

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 73 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Ala Arg Val Arg Phe Ser Pro His Val Arg Val Arg His Leu Val Val

75

1	5	10	15
Trp Ala Ser Ala Ala Arg Leu Ala Arg Arg Gly Ser Trp Ala Arg Glu			
20	25	30	
Arg Ala Asp Arg Ala Arg Phe Arg Arg Arg Val Ala Glu Ala Glu Ala			
35	40	45	
Val Ile Gly Pro Cys Leu Gly Lys Glu Ala Arg Ala Arg Ala Leu Ala			
50	55	60	
Arg Gly Ala Gly Pro Ala Asn Ser Val			
65	70		

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Asp Glu Tyr Asp Asp Ala Ala Asp Ala Ala Gly Asp Arg Ala Pro			
1	5	10	15

Gly Met

WHAT IS CLAIMED IS:

1. A method of preventing or treating programmed cell death in neuronal cells, the method comprising:
 - (a) preparing a non-pathogenic vector comprising the γ ,34.5 gene; and
 - (b) introducing the non-pathogenic vector into neuronal cells undergoing or likely to undergo programmed cell death.
2. The method of claim 1 wherein the vector comprises an HSV-1 or HSV-2 virus altered in such a way as to render it non-pathogenic.
3. The method of claim 2 wherein said alteration comprises deletion of the ICP4 gene, the α 4 gene, or the α 0 gene of the HSV-1 or HSV-2 viral genome.
4. The method of claim 2 wherein said alteration comprises a mutational lesion of the ICP4 gene, the α 4 gene, or the α 0 gene of the HSV-1 or HSV-2 viral genome.
5. The method of claim 1 wherein said vector comprises a retrovirus altered, a vaccinia virus, a picornavirus, a coronavirus, a eunyavirus, a togavirus, or a rhabdovirus in such a way as to render it non-pathogenic.
6. The method of claim 1 wherein the vector comprises a multipotent neural cell line.
7. The method of claim 1 further comprising introducing the vector into the neuronal cells undergoing or likely to undergo programmed cell death by a process comprising transplanting cells of the multipotent neural cell line of claim 6 into a region of the central nervous system in which said neuronal cells undergoing or likely to undergo programmed cell death are located.

8. The method of claim 1 further comprising introducing the vector into neuronal cells of an animal by injection of the vector at the site of the peripheral nerve endings of the neuronal cells undergoing or likely to undergo cell death.

9. The method of claim 1 further comprising introducing the vector into neuronal cells in culture likely to undergo or undergoing cell death by incubation of the vector with the neuronal cells.

10 10. A viral vector comprising the HSV-1 or HSV-2 virus having a genomic alteration rendering the viral vector non-pathogenic.

11. The viral vector of claim 10 wherein said genomic alteration comprises deletion of the ICP4 gene, the $\alpha 4$ gene, or the $\alpha 0$ gene of the HSV-1 or HSV-2 genome.

12. The viral vector of claim 10 wherein said genomic alteration comprises a mutational lesion of the ICP4 gene, the $\alpha 4$ gene, or the $\alpha 0$ gene of the HSV-1 or HSV-2 genome.

13. A viral vector comprising a non-pathogenic retrovirus, vaccinia virus, picornavirus, coronavirus, eunyavirus, togavirus, or rhabdovirus.

14. A method of preventing or treating programmed cell death in neuronal cells, the method comprising:

25 (a) preparing ICP34.5 or a biological functional equivalent thereof;

(b) combining the ICP34.5 or the biological functional equivalent with a pharmaceutically acceptable carrier to form a pharmaceutical composition; and

(c) administering the composition to neurons likely to undergo or undergoing programmed cell death.

15. The pharmaceutical composition of claim 14, said pharmaceutical composition comprising ICP34.5 or a
5 biological functional equivalent thereof in a pharmaceutically acceptable carrier.

16. The method of claim 14 wherein ICP34.5 or its biological functional equivalent is prepared by the method comprising:

10 (a) preparing a nucleic acid segment capable of encoding ICP34.5 or a biological functional equivalent; and

(b) expressing the segment to produce the ICP34.5 or biological functional equivalent protein.

15 17. The method of claim 16 wherein the segment is transferred into a host cell and the host cell is cultured under conditions suitable for expression of the segment.

20 18. The method of claim 17 wherein the nucleic acid segment is transferred by transfection or transformation of a recombinant vector into the host cell.

19. The method of claim 17 further comprising isolating and purifying the protein.

25 20. The method of claim 14 wherein the composition is administered to an animal by direct, intrathecal or intravenous injection, or by oral administration.

21. The method of claim 14 wherein the composition is administered to neuronal cells in culture by incubation of

said cells in a medium comprising the composition such that the protein will enter the cells.

22. A method for determining the ability of a candidate substance to protect cells from programmed cell death, said method comprising:

- (a) preparing a neuronal cell line sensitive to programmed cell death;
- (b) combining the cells of said cell line with the candidate protective substance;
- 10 (c) altering the incubation solution such that the cells are exposed to conditions or substances capable of inducing programmed cell death; and
- (d) determining whether the candidate substance has protected the cells from programmed cell death.

15 23. The method of claim 22 wherein the candidate substance is a putative biological functional equivalent of ICP34.5.

24. A method for determining the ability of a candidate substance to potentiate the protective function 20 of ICP34.5 or biological functional equivalents thereof, said method comprising:

- (a) preparing a neuronal cell line sensitive to programmed cell death;
- (b) combining the cells of said cell line with the candidate potentiating substance;
- 25 (c) adding to the incubation medium ICP34.5 or a biological functional equivalent thereof;

(d) altering the incubation solution such that the cells are exposed to conditions or substances capable of inducing programmed cell death; and

5 (e) determining whether the candidate substance has potentiated the protective effects of ICP34.5 or the biological functional equivalent.

10 25. A method for determining the ability of a candidate substance to act as an inhibitor of either γ ,34.5 expression or activity of ICP34.5 or biological functional equivalents thereof, said method comprising:

(a) preparing a neuronal cell line sensitive to programmed cell death;

(b) combining the cells of said cell line with the candidate inhibitory substance;

15 (c) adding to the incubation medium ICP34.5 or a biological functional equivalent thereof;

(d) altering the incubation solution such that the cells are exposed to conditions or substances capable of inducing programmed cell death; and

20 (e) determining whether the candidate substance has inhibited the protective effect of ICP34.5 or the biological functional equivalent.

26. A method of delivering a gene for gene therapy, the method comprising:

25 (a) combining the gene for gene therapy with any one of the vectors of claims 10, 11, 12 or 13;

(b) combining the gene and vector with a pharmacologically acceptable carrier to form a pharmaceutical composition; and

5 (c) administering said pharmaceutical composition so that the gene and vector will reach the intended cell targets.

27. The method of claim 26 wherein said pharmaceutical composition is introduced by injection into an animal at the site of said cell targets.

10 28. The method of claim 26 wherein said cell targets are in the central nervous system and the pharmaceutical composition is introduced by injection into an animal at the site of the peripheral nerve ending which originate from neurons located at the site of said cell targets.

15 29. The method of claim 26 wherein said pharmaceutical composition is introduced by intrathecal or intravenous injection.

20 30. The pharmaceutical composition of claim 26 comprising the gene and vector combined with a pharmacologically acceptable carrier.

31. A method of treating tumorogenic diseases or viral infections, the method comprising:

(a) preparing a candidate substance of claim 25;

25 (b) combining the candidate substance with a pharmaceutically acceptable carrier to form a pharmaceutical composition; and

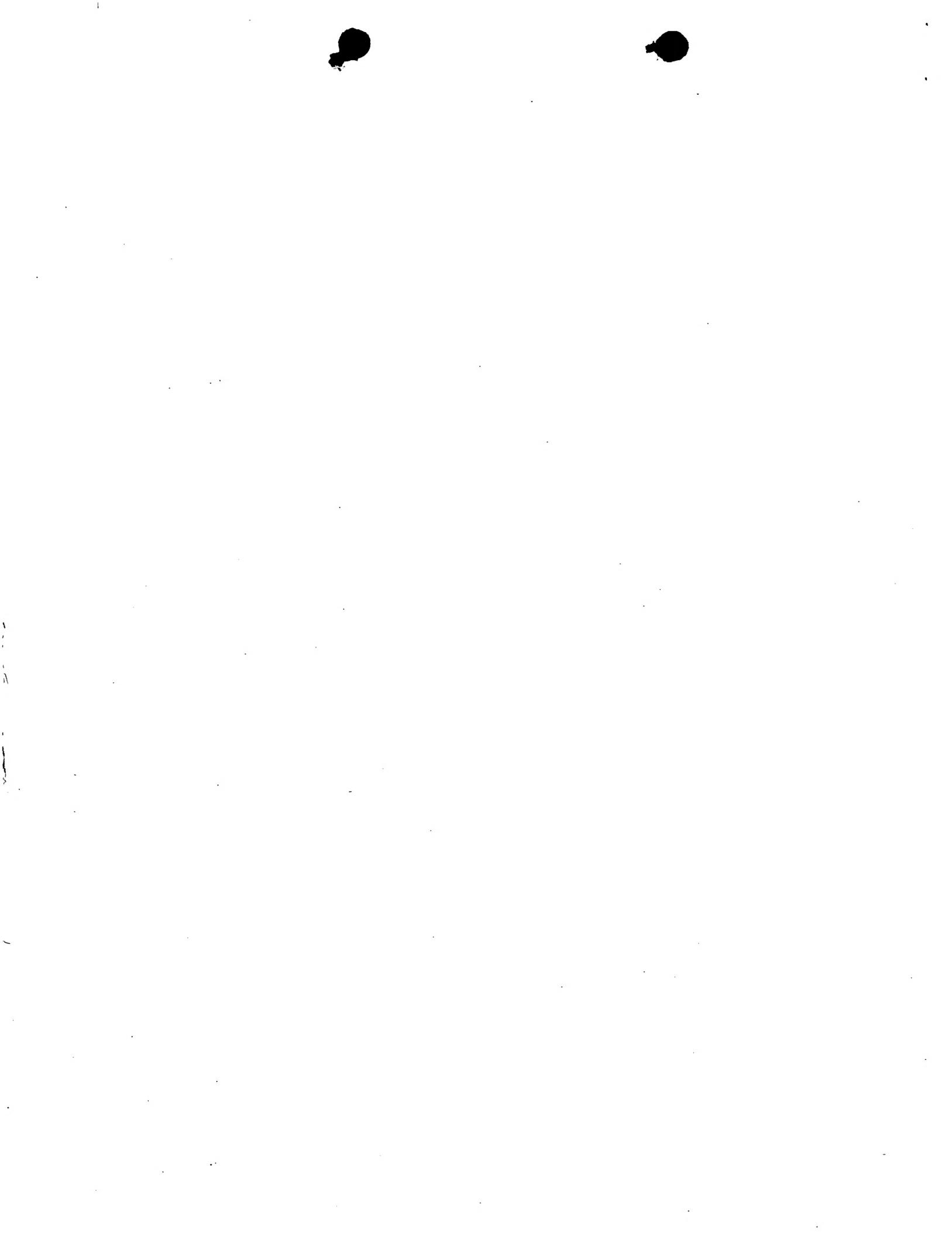
(c) administering the composition to tumor cell targets.

32. The method of claim 31 wherein the pharmaceutical composition is administered by injection directly into the site of the cell targets, by intravenous injection, by intraspinal injection, or orally.

- 5 33. The pharmaceutical composition of claim 31 comprising the candidate substance combined with a pharmaceutically acceptable carrier.

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FIG. 1A



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FIG. 1B



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FIG. 1C

MetAlaArgArgArgArg---HisArgGlyProArgArgProArgProProGlyProThrGlyAlaValProIthr
24

Arg

АГ

AlaGlnSerGlnValThrSerThrProAsnSerGluProAlaValArgSerAlaproAlaAlaAlaAlaProProPro

Val

PROPOSALS ARE APPROVED BY THE BOARD OF TRUSTEES.

Gly
Gly
Gly
Gly

ASP ASP ASP ASP ASP ASP ASP Trp Pro Asp Ser Pro Pro Glu Ala Pro Glu Ala Arg Pro Thr Ala Ala Ser

**AlaProArgproArgSerproProGlyAlaGlyProGlyGlyGlyAlaAsnProSerHisProProSerArg
ProGlyProHisArgProAlaTrpAlaArgGlyAlaGlyLeuThrProProThrProProArg**

ProPheArgLeuProProArgLeuAlaLeuArgValThrAlaGluHisLeuAlaArgLeuArg
AlaProSerAlaPheArgGargAlaSerProSerAlaCysAlaSerProArgSerThrTrpArgAlaCysAlaCys

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ArgAlaGlyGluGlyAlaProGluProProAlaIhrProAlaIhrProAlaIhr
AspAlaArgAlaGlyArgGlyArgArgSerProProArgPrcProArgProArg
Lys

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FIG. 1D

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64

ProProArgLeuProProArgLeuAlaLeuArgLeuArgValThrAlaGluHisLeuAlaArgLeuArg
 AlaProSerAlaPheArgArgAlaSerProSerAlaCysAlaSerProArgSerThrIrrPheArgAlaCysAlaCys
 ArgAlaGlyGluGlyAlaProGluProProAlaThrProAlaThrProAlaThrProAlaThr
 AspAlaArgAlaGlyArgGlyArgArgSerProProArgPrcProArgProArgProProArg
 Lys

* * * * *
ProAlaThrProAlaThrProAlaThrProAlaThrProAlaArgValArgPheSerProHisVal
Pro-----ProArgGlyCysAlaSerArgProThr

ArgValArgHisLeuValValTrpAlaSerAlaAlaArgLeuAlaArgArgGlySerTrpAlaArgGluArgAla
SerGlyCysAlaThrTrpSerGlyProArgProProAlaTrpArgAlaAlaArgGlyProAlaSerGly

AspArgAlaArgPheArgArgArgValAlaGluAlaGluAlaValIleGlyProCysLeuGlyProGluAlaArg
ProThrGlyLeuGlySerGlyIlaGlyTrpArgArgProArgSerSerGlyArgAlaTrpGlyProArgPro

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AlaArgAlaLeuAlaArgGlyAlaGlyProAlaAsnSerVal0c
ValProGlyProTrpProAlaGluProAlaArgArgThrArgSerAsnValThrProGluAlaAlaItpValPhe

ArgGlyAlaProGlySerSerAlaProSerArgSerProGluArgArgIrpGlnGluProArgIleTyrThrLeu

NaAgroProfilSerVaArgArAvAlProfesionalVarasenelProyectoYaguanay

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FIG. 2

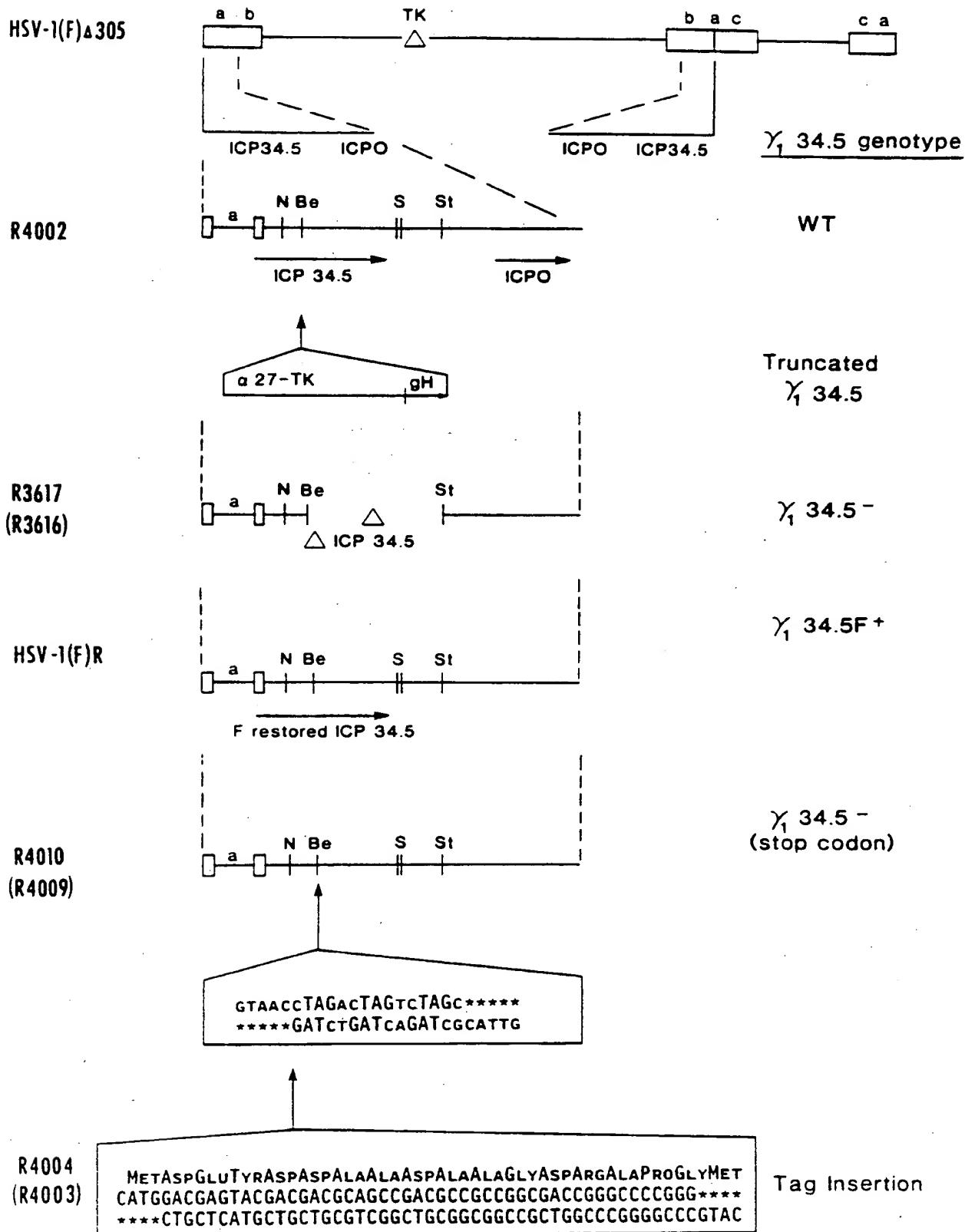
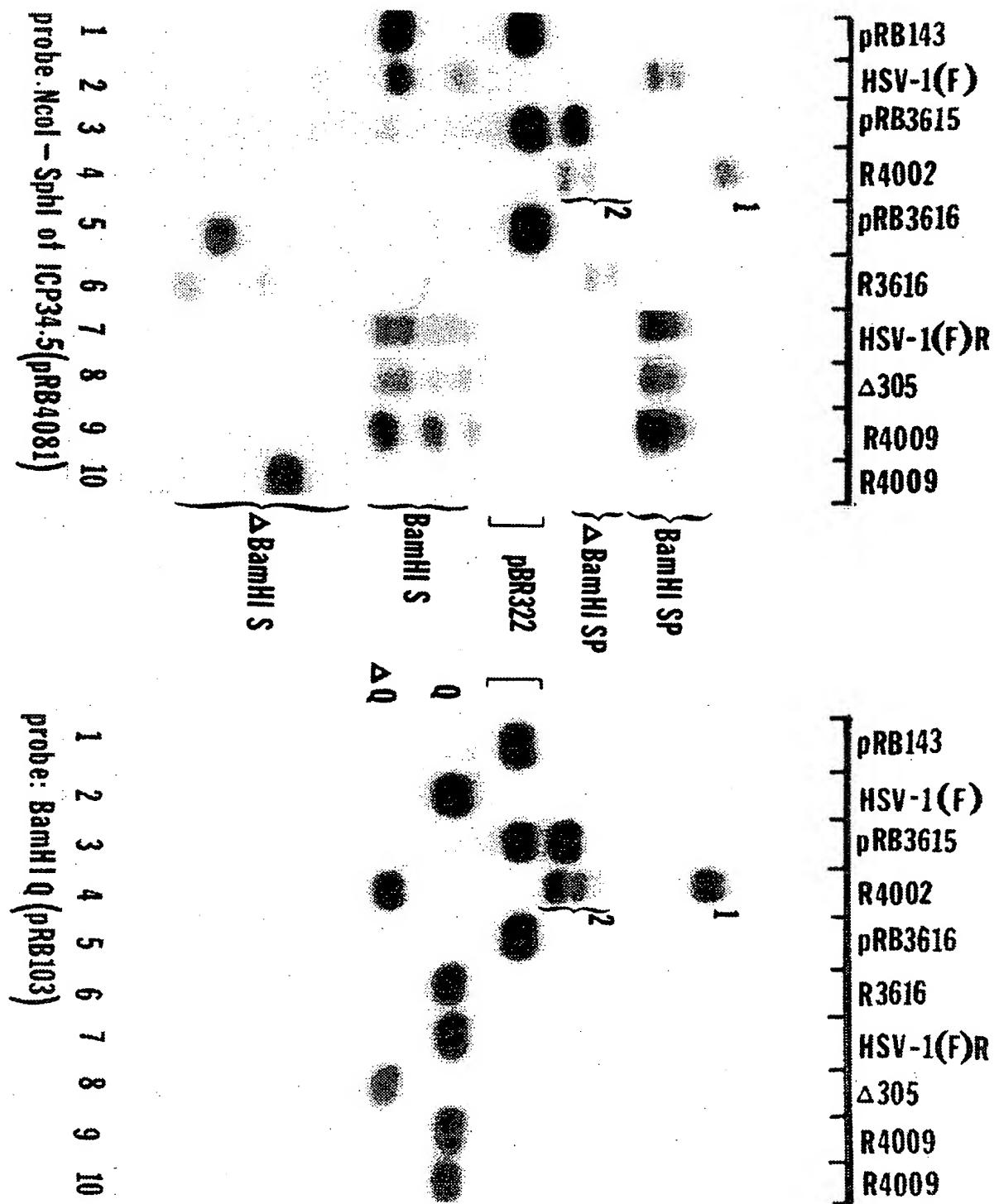


FIG. 3



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FIG. 4

³⁵S-labeled
Ab: rabbit anti(Ala-Thr-Pro)₁₀

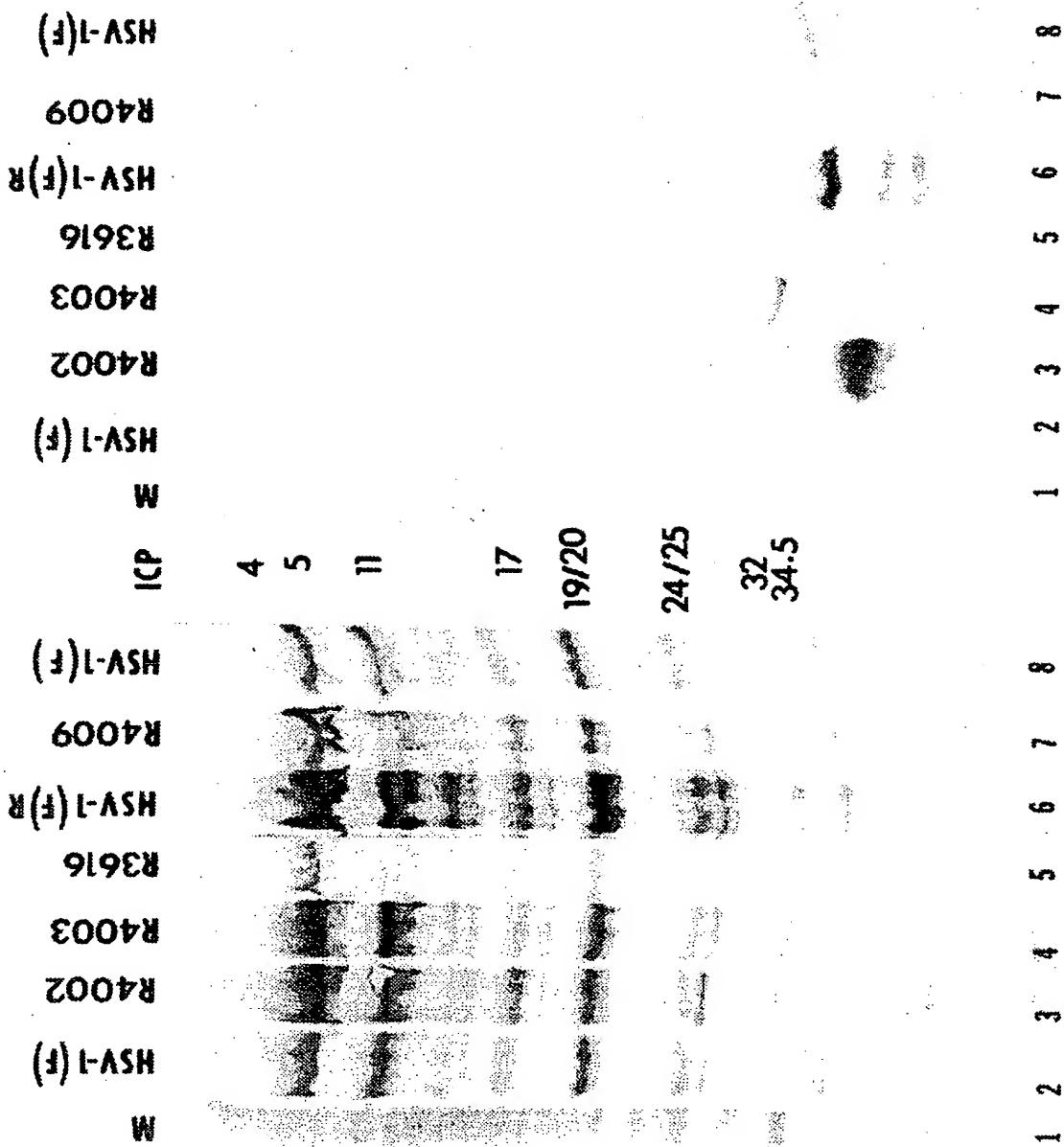


FIG. 4

FIG.5

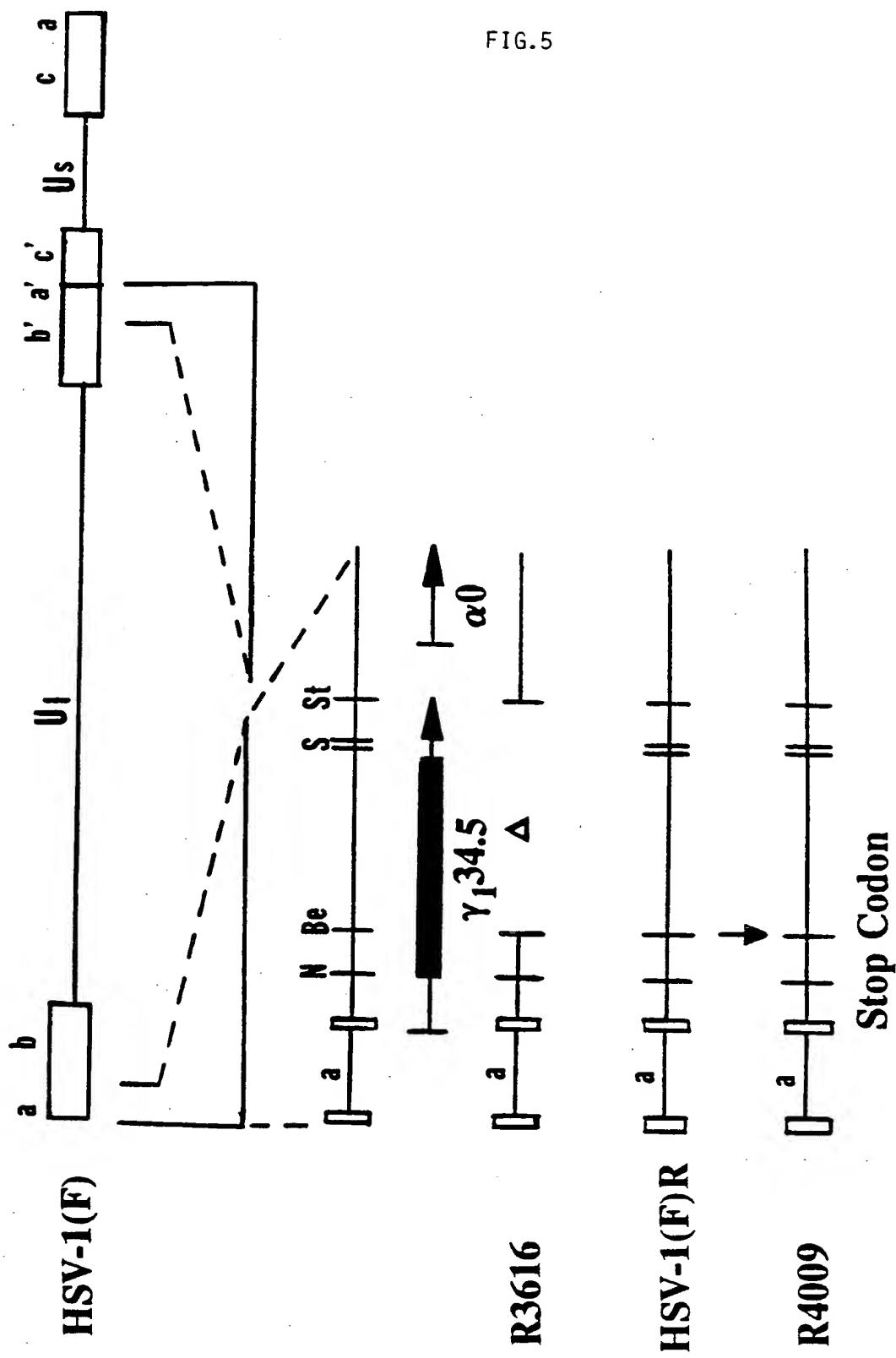
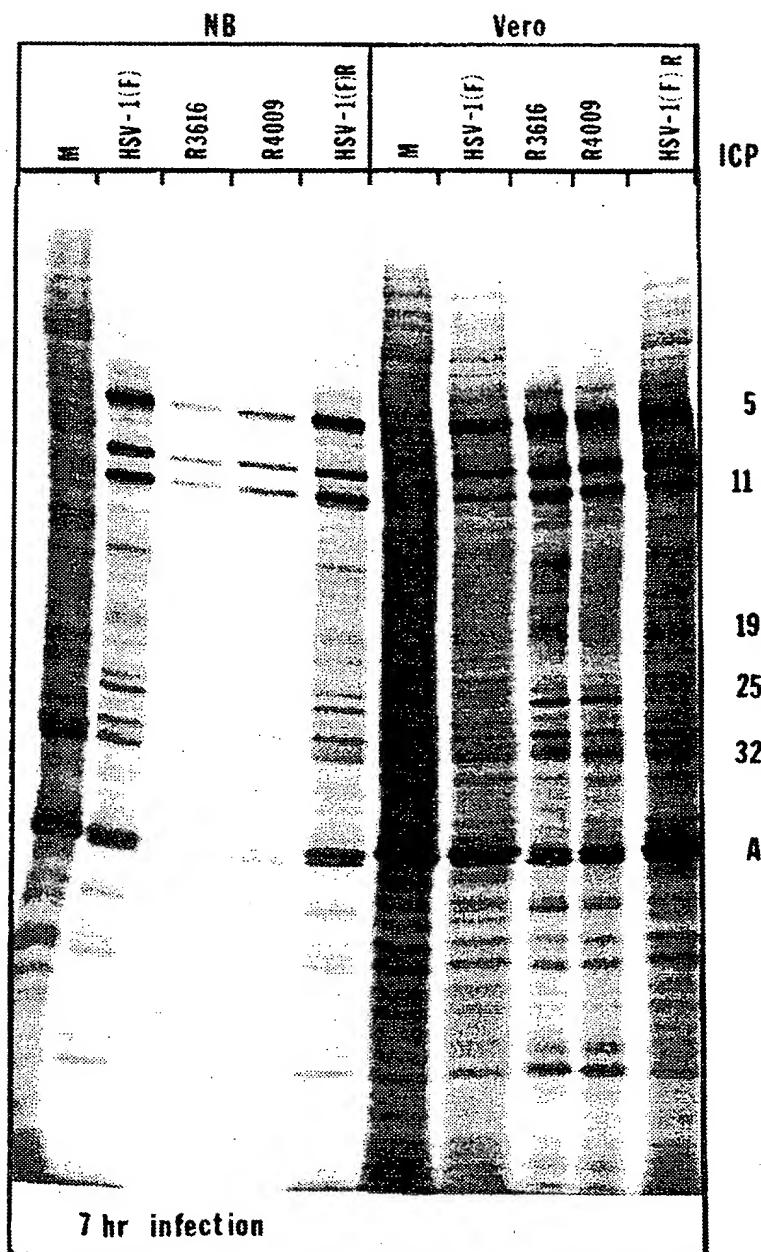


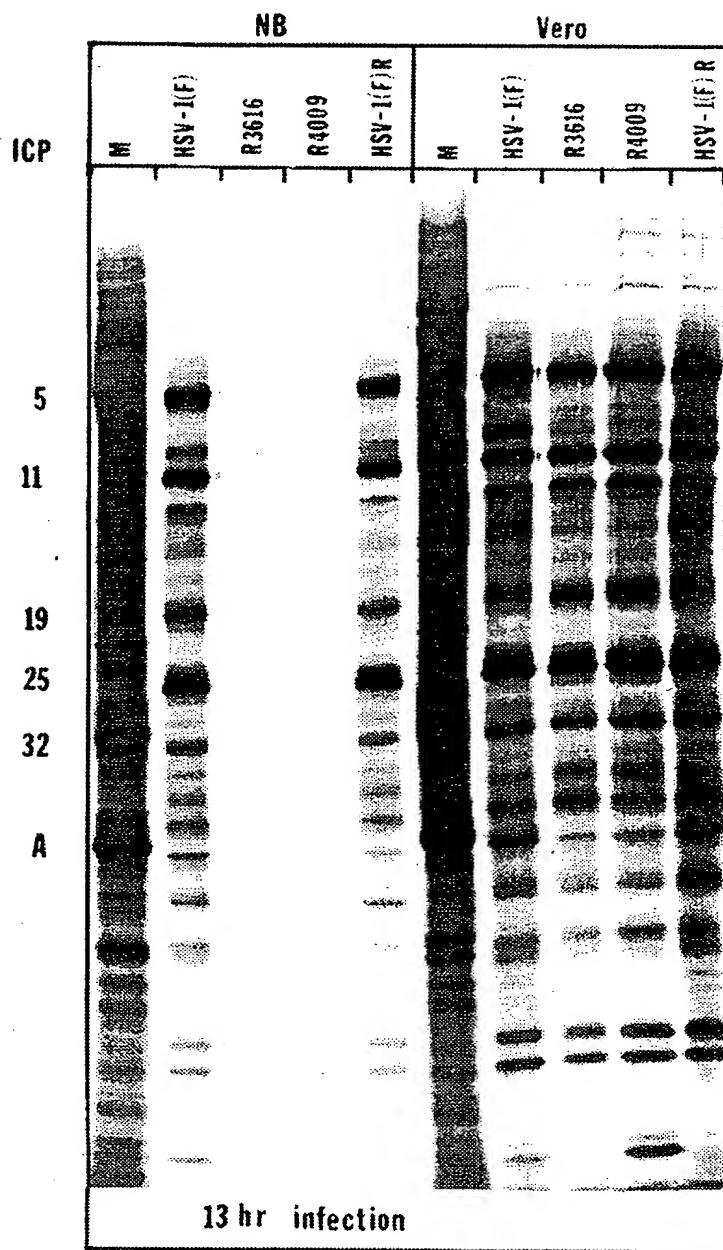
FIG. 6 A



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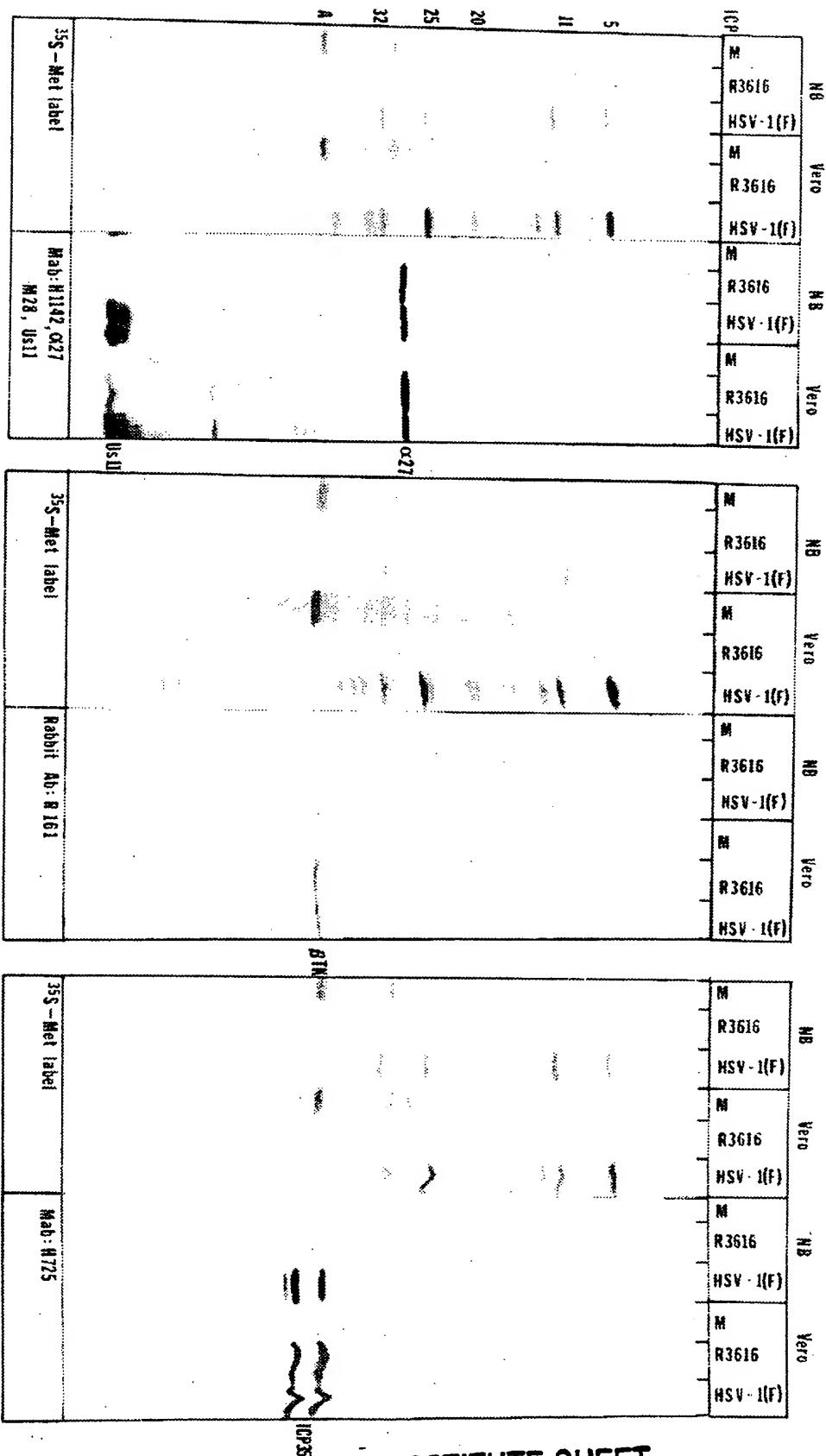
FIG. 6B



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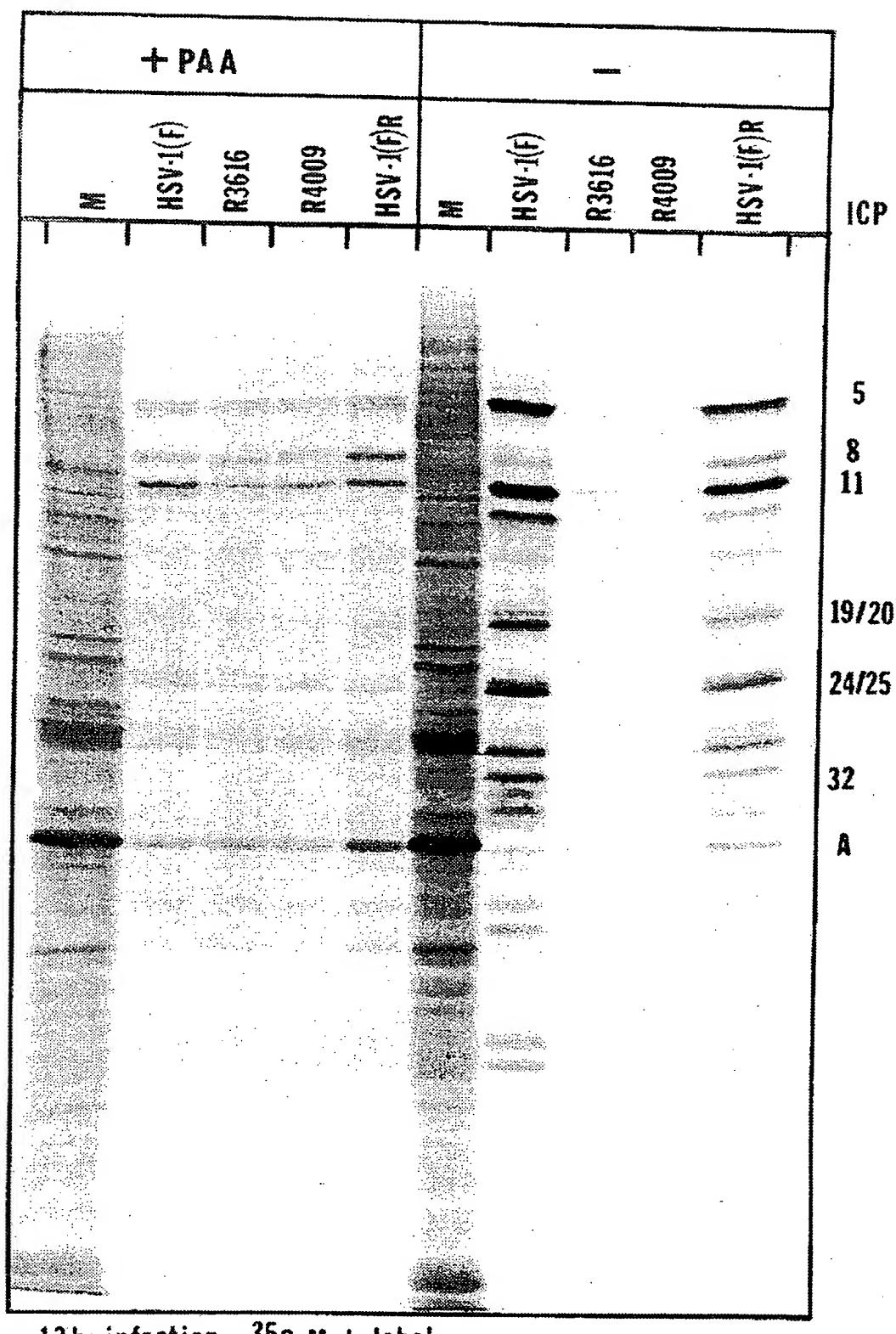
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FIG. 7



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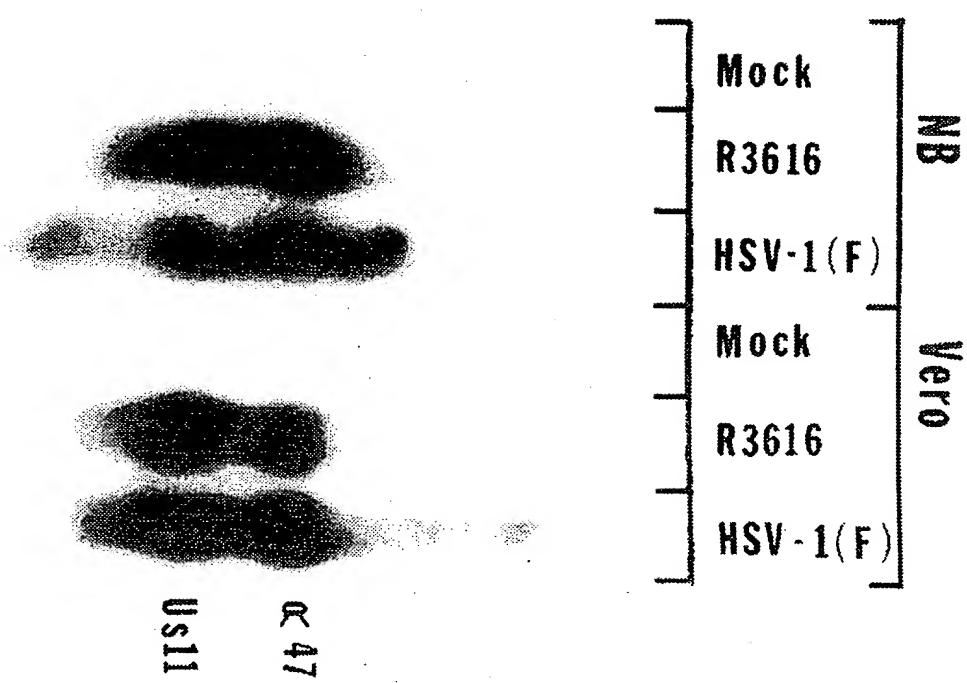
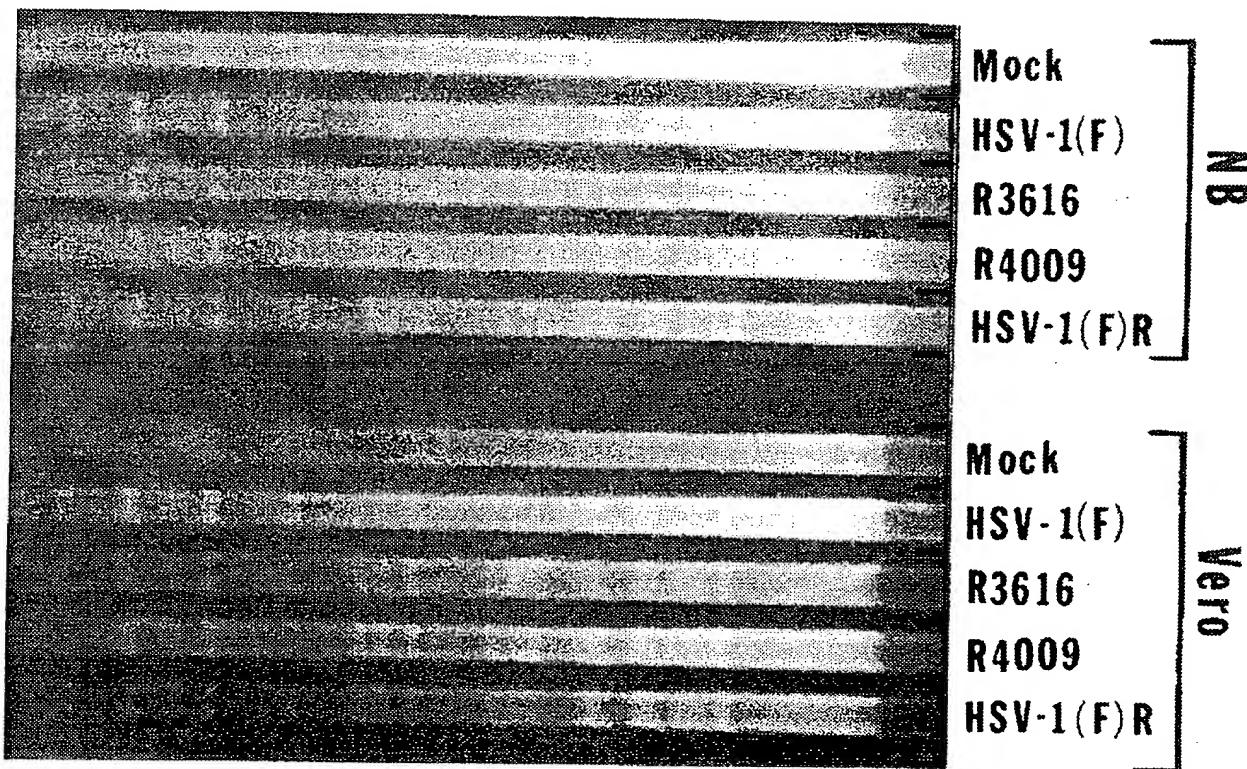
FIG. 8





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FIG. 9



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/01801

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01K 63/00; A61K 37/00, 31/70; A01N 37/18; C12N 15/00; C12Q 1/02;
 US CL :424/93B; 435/29, 320.1; 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93B; 435/29, 320.1; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chem. Abs.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 65, issued 15 December 1991, S. Henderson, "Induction of <u>bcl-2</u> Expression by Epstein-Barr Virus Latent Membrane Protein 1 Protects Infected B Cells from Programmed Cell Death", pages 1107-1115, see entire document.	1-33
Y	Cell, Volume 67, issued 29 November 1991, A. Strasser et al, " <u>bcl-2</u> Transgene Inhibits T Cell Death and Perturbs Thymic Self-Censorship", 889-899, see entire document.	1-33

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

04 June 1993

Date of mailing of the international search report

14 JUN 1993

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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/01801

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Vol. 67, issued 29 November 1991, C.L. Sentman et al, " <u>bcl-2</u> Inhibits Multiple Forms of Apoptosis but Not Negative Selection in Thymocytes", pages 879-888, see entire document.	1-33
Y	Nature, Vol. 349, issued 14 February 1991, C. D. Gregory et al, "Activation of Epstein-Barr Virus Latent Genes Protects Human B Cells From Death by Apoptosis", pages 612-614, see entire document.	1-33